METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF ACECLOFENAC, PARACETAMOL & CHLORZOXAZONE IN TABLETS BY HPLC, HPTLC & ACECLOFENAC BY DIFFERENTIAL SPECTROSCOPY

A Dissertation submitted to
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Chennai

In partial fulfillment of the requirements
for the award of the degree of

MASTER OF PHARMACY
IN
PHARMACEUTICAL CHEMISTRY

Submitted by
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SEPTEMBER 2007

CERTIFICATE

*This is to Certify that this dissertation entitled "**Method Development and Validation for simultaneous estimation of aceclofenac, paracetamol & chlorzoxazone in tablets by HPLC, HPTLC & Aceclofenac by differential spectroscopy**" submitted by the candidate **T. Venkatesh Karthikeyan** for the award of **M.Pharm degree** is a bonafide record of the research work done by her under my guidance and supervision during his course of study at Madras Medical College, Chennai-3 and that it has not previously formed the basis for the award of any degree or any other similar title and that it is an independent work done by him.*

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CONTENTS

LIST OF ABBREVIATIONS

1. HPLC - High Performance Liquid Chromatography
2. HPTLC - High Performance Thin Layer Chromatography
3. UV - Ultra Violet Spectroscopy
4. LOD - Limit of Detection
5. LOQ - Limit of Quantification
6. mg/ml - milligram / milliliter
7. µg/ml - micro gram / milliliter
8. ng/ml - nano gram / milliliter

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INTRODUCTION

The number of drugs and formulations introduced into the market is increasing every year. It is therefore, necessary to develop newer analytical methods for such drugs and formulations. The reasons for the development of newer methods of drug analysis are:-

The drug or drug combination may not be official in any pharmacopoeia. A proper analytical procedure for the drug may not be available in the literature due to patent regulations.

- Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients.
- Analytical methods for the quantification of the drug in biological fluids may not be available.
- Analytical methods for a drug in combination with other drugs may not be available.

- The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable.

Estimation of drugs in single and multi component formulations:

Drugs are typically developed and manufactured into formulations prior to their use by patients. Very often administration of two or more drugs at a time becomes imperative for several therapeutic reasons and there exist a number of drug combinations which have proved to have better therapeutic effect due to their synergistic effect, ease of administration as a single dose which also has a higher patient compliance and economy in production, distribution and treatment costs.

There are several methods of analysis of formulations, which include

a) Classical separation and analysis (non-instrumental):

It involves the separation of components of interest using the classical separation techniques like extraction or isolation and then a suitable estimation procedure is selected to quantify the individual components. It includes the techniques like gravimetry, volumetry, etc.

b) Spectral Methods

In spectral techniques, the electro magnetic radiation that is either absorbed or emitted by the sample is measured. It includes the techniques like UV-visible spectroscopy, infrared spectroscopy, flame photometry, fluorimetry, etc.

c) Electro Analytical Methods

It involves the measurement of current, voltage or resistance as a property of the concentration of the drug in solution mixture. It includes the techniques like potentiometry conductometry, amperometry, etc.

d) Chromatographic Methods

The different chromatographic techniques available are thin layer chromatography, gas chromatography, column chromatography, paper chromatography, size exclusion chromatography, high performance liquid chromatography and high performance thin layer chromatography. The most reliable and widely used advanced chromatographic techniques for the estimation of drug in formulation are.

i) Gas liquid chromatography (GLC)

In this technique, a carrier gas is used as the mobile phase and it passes over a stationary phase which is a non volatile liquid coated on an inert solid support. Separation takes place in accordance with the partition co-efficients of the components in the mixture.

ii) ***High performance Liquid chromatography (HPLC)***

The technique of high performance liquid chromatography is so called because of its improved performance when compared to classical column chromatography.

iii) High Performance thin layer chromatography (HPTLC)

It is a sophisticated, advanced and automated version of thin layer chromatography.

OBJECTIVE OF THE STUDY

The number of drugs and drug formulations introduced into the market is increasing at an alarming rate. The complexity of these dosage forms poses considerable challenge to the analytical chemist during the development of assay procedure. The estimation of individual drugs and multi component dosage forms become difficult due to cumbersome extraction and isolation procedure.

For the estimation of the drugs present in formulations, methods like HPLC, HPTLC and spectrophotometric methods are more suitable since these methods are highly specific, linear, precise, accurate, sensitive and rapid.

From the review of literature, we found no documentary evidence is available for the quantitative analysis of ternary mixture, Aceclofenac, paracetamol and chlorzoxazone. We also found that there is no reported method for the estimation of drugs by differential spectrometry.

In the proposed work, we developed HPLC and HPTLC method to quantify aceclofenac in combination with paracetamol and chlorzoxazone and individually using differential spectroscopy.

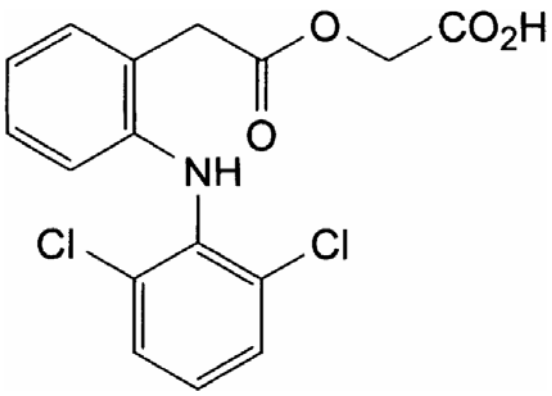
DRUG PROFILE

ACECLOFENAC

Aceclofenac is an analgesic and anti-inflammatory agent used to relive pain associated with inflammation.

CHEMISTRY:

Molecular formula : $C_{16}H_{13}NO_4Cl_2$



Chemical Name : [(2-[(2,6-dichlorephenyl) amino] phenyl] acetyl]oxy]acetic acid.

Molecular weight : 354.2

Description : white or almost white crystalline powder

Solubility : Practically insoluble in water, freely soluble in acetone, soluble in alcohol.

This drug is official in B.P 2007 Volume –I

Mechanism of Action:

Aceclofenac has been shown to exert effects on many mediators of inflammation. It inhibits the synthesis of inflammatory cytokinines, interleukin – 1b and tumour nerosis factor and inhibits prostaglandinE_2 production. It also stimulates glycosaminoglycans (GAG) synthesis, which aids in repair and regeneration of articular damage.

Indications:

Management of osteoarthritis, rheumatoid arthritis and alkylyosing spondylitis.

Dose:

Adults 100 mg b.i.d.

CHLORZOXAZONE

Chlorzoxazone is a centrally acting skeletal muscle relaxant.

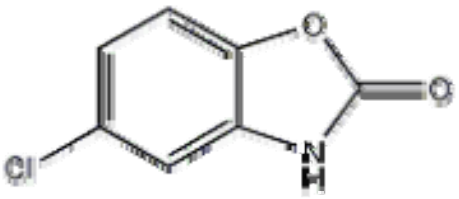
Chemistry

Molecular formula : $C_7H_4Cl\ NO_2$

Chemical name : 5-chloro-2-benzoxazolinone.

Molecular weight :169.56

Chemical Structure :



Description : It is a white or almost white crystalline powder

Solubility : it is freely soluble in methanol and soluble in water and acetonitrile.

This durg is official in USP / NF – 2006, EP Volume.2 01 /2005ble

Mechanism of action:

Chlorzoxazone is a centrally acting muscle relaxant for painful musculoskeletal conditions. It acts at the level of the spinal cord and subcortical areas of the brain where it inhibits multisynaptic areas involved in producing and maintaining skeletal muscle spasm of varied etiology. It also possesses sedative property.

Indication:

Adjuvant in the treatment of short-term symptomatic treatment of painful muscle spasm associated with musculoskeletal conditions.

Dose:

Adults: initially 500 mg, t.i.d. or q.i.d. subsequently reduced to 250 mg t.i.d./q.i.d.

PARACETAMOL

Paracetamol is an analgesic and antipyretic agent.

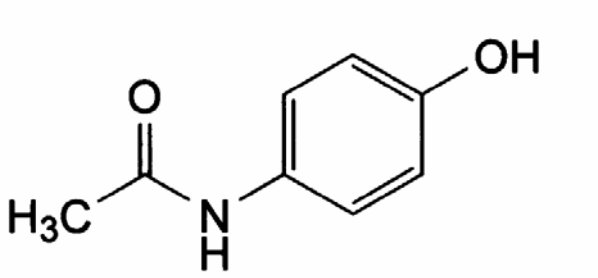
Chemistry:

Molecular formula : $C_8H_9NO_2$

Molecular weight : 151.2

Chemical name : N-(4-hydroxyphenyl) acetamide.

Chemical structure :



This drug is official in BP -2007 volume –II, USP / NF 2006 and EP 5.0 Volume 2 01 / 2005.

Description : White crystalline powder

Solubility : Sparingly soluble in water, freely soluble in alcohol, very slightly soluble in methylene chloride.

Mechanism of Action:

Paracetamol exhibits analgesic action by peripheral blockade of pain impulse generation. It produces antipyretic effect by inhibiting the hypothalamic temperature regulating centre. It's weak anti-inflammatory activity is related to inhibition of prostaglandin synthesis in the CNS.

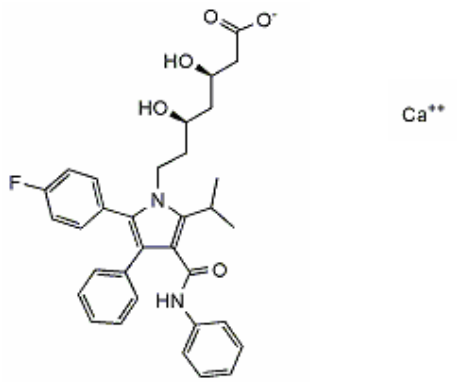
Indication : Relief of mild to moderate pain & fever

Dose : Adults 0.5-1g every 4-6 hours Max dose 4g daily

Childrens: 6 -12years 250-500mg 4-6hours

ATORVASTATIN

Molecular formula : 2 C_{33} H_{34} F N_2 O_5. Ca



Chemical Name : (β, R, δR) - 2 (4 fluoro)

Phenyl, β, δ, dihydroxy - 5.5 - 1 methyl

ethyl 3 - phenyl - 4 − (phenyl amino) carboxy 1-H - Pyrrole 1 -

heptonoic acid

Solubility : Insoluble in aqueous solutions of PH4 and below

Very slighty soluble in distilled H20. PH 7.4 and acetonitrile

Mechanism of Action:

It competitively inhibit HMG - CoA reductase the enzune which converts HMG - CoA to mevalonic acid. This is the rate limiting step in the biosynthesis of cholesterol. Thus it lowers the LDL level.

Indication

In the treatment of hyper cholestermia

REVIEW OF LITERATURE

Chatterjee. P.K. et.al (1989) performed the simultaneous determination of chlorzoxazone and acetaminophen in combined dosage forms by an absorbance ratio technique and difference spectrophotometry.

Pank S.K. et al (1990) carried out the simultaneous determination of oxyphenbutazone, chlorzoxazone and paracetamol in dosage forms by RP-HPLC.

El.Kousy et, al (1994) proposed potentiometric & spectrocolourimetric methods for the estimation of chlorzoxazone in the presence of paracetamol.

Bhatha.Ms(1995) proposed the simultaneous spectrophotometric determination of diclofenac sodium, chlorzoxazone and paracetamol from combined dosage forms.

Sodhi.R.A (1996) carried out the simultaneous determination of paracetamol, ibuprofen and chlorzoxazone by HPLC, HPTLC and GLC methods

El-Kousy–NM (1999) developed spectrophotometric and spectorfluorimetric determination of etodolac and aceclofenac.

Lec H.S. et.al (2000) carried out simultaneous determination of aceclofenac and diclofenac in human plasma by narrowbore HPLC using column switching

Rajnarayana K. et.al(2002) validated a HPLC method for determination of chlorzoxazone in human serum and it's application in a clinical pharmacokinetic study.

Zawilla –N.H et.al (2002) performed the determination of aceclofenac in bulk and pharmaceutical formulations.

El-saharty et.al (2002) proposed stability indicating spectrophotometric and densitometric methods for determination of aceclofenac.

Lin–HM. Et.al(2003) carried out HPLC determination of the concentration of aceclofenac in human plasma.

Wahbi – AA. Et.al (2003) performed the simultaneous determination of paracetamol and chlorzoxazone using orthogonal functions ratio spectrophotometry.

Zhang-HS. et.al (2003) reported the simultaneous determination of paracetamol and caffeine is fufangan fenwanen capsules by HPLC.

Acuna J.A et.al (2004) studied the polarographic behaviour of aceclofenac, Jenoxicam and Droxicam in a methanol –water mixture.

Zinellu A.et.al (2005) carried out the separation of aceclofenac & diclofenac in human plasma by free zone capillary electrophoresis using Methyl –D- glutamine as an effective electrolyte additive.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC has its origin in the early 1960s and was developed based on the experience gained from both gas chromatography and column chromatography. It was first marketed by Dupont and it then leads to rapid development of HPLC as a routine analytical tool.

The technique of HPLC is based on the same modes of separation as column chromatography i.e. adsorption, partition, including reverse phase partition, ion-exchange and gel permeation. But this method differs from column chromatography by pumping the mobile phase at high pressure. The advantages of HPLC are improved resolution of the separated substances, faster separation times and the increased accuracy, precision and sensitivity with the substances being quantified.

ADSORPTION HPLC:

It is also generally referred to as normal phase chromatography. Normal phase chromatography is based on polar stationary phases. The mobile phase consists of a non-polar solvent.

REVERSE PHASE HPLC:

In reverse phase chromatography the stationary phase is prepared by chemically bonding a relatively non-polar group onto the stationary phase support. The most frequent non-polar group bonded to the stationary support is octadecyl silane (ODS or C18) which gives a highly lipophilic stationary phase. Less lipophilic stationary phases are produced when octylsilane (C8), C2, phenyl or cyanopropyl bonded stationary phases are used.

The mobile phase in reverse phase HPLC is polar, generally consisting of water and water-miscible organic solvent, such as methanol or acetonitrile.

Separation in these system is based on different degrees of lipophilicity of the solutes; the polar components gets eluted first and non-polar components are retained for a longer time.

Ion-exchange HPLC:

The ion exchangers in HPLC are generally silica stationary phases chemically bonded with anionic or cationic groups, usually aminopropyl, tetra-alkyl ammonium or sulphonic acid groups. Molecules are generally separated on the basis of their molecular charge

on the principle of opposite charges attract each other. Resolution is influenced by pH and by ionic strength of the buffer.

Size exclusion chromatography:

The separation is based on the size and molecular weight. The stationary phase is generally a semi-rigid, porous material in which the pore size is selected is selected to be similar to that of solute particles. The mobile phase plays no role in the separation, the only requirement being that the solutes are soluble in it.

INSTRUMENT EMPLOYED:

Chromatographic instrument	: Shimadzu LC-10 AT VP
Chromatographic column	: Phenomenex, Luna 5 micron C18, Size – 250 x 4.60 mm.
Detector	: Shimadzu SPD-10 AVP
Injection volume	: 20 µl
Syringe	: Hamilton 25 µl syringe
Mode of operation	: Isocratic
Temperature	: Ambient
Flow-rate	: 1.5 ml/min
Filter membrane	: Ultipor N66 (nylon 6, 6 membrane) From PALL life sciences.
Balance	: Mettler AB54
pH meter	: Digital pH meter from Md.dalal.

CHEMICALS:

Aceclofenac from Paris-Dakner Pharmaceuticals, Chennai.

Paracetamol from Paris-Dakner Pharmaceuticals, Chennai.

Chlorzoxazone from Paris-Dakner Pharmaceuticals, Chennai.

Atorvastatin from Paris-Dakner Pharmaceuticals, Chennai.

Acetonitrile HPLC grade from E-Merck.

Methanol HPLC grade from E-Merck.

Water HPLC grade from E-Merck.

Ortho-phosphoric analytical grade from Sd fine chemicals

Disodium hydrogen orthophosphate from sd fine chemicals.

REAGENTS:

Stock solution:

Standard stock solutions containing 1mg/ml of aceclofenac, 5 mg/ml of chlorzoxazone, and 5 mg/ml of paracetamol are prepared in methanol.

Preparation of mobile phase:

Buffer solution:

Dissolved 3.55 grams of disodium hydrogen ortho phosphate in water and made upto 500 ml with water.

Mobile phase:

Taken 325 ml of acetonitrile and 175 ml of buffer solution and the pH was adjusted to 3 with ortho phosphoric acid. The mobile phase is then filtered through the Ultipor Nylon 66 membrane.

Establishment of R_t:

For determining the R_t values, working standard solutions were prepared taking 2 ml of the standard solutions of aceclofenac, paracetamol and chlorzoxazone in a series of volumetric flask and made up to 10 ml with mobile phase. Diluted 3 ml of these solutions in a series of 10 ml of volumetric flask to obtain 60 µg/ml of aceclofenac and 300 µg/ml of paracetamol and chlorzoxazone. Injected 20 µl of these solutions into the rheodyne injector (272 nm) and the R_t values for the paracetamol, aceclofenac and chlorzoxazone are established as given below

The retention times are,

Paracetamol : 1.85

Chlorzoxazone : 3.01

Aceclofenac : 3.82

.

Optimization of chromatographic conditions:

1. ***Selection of chromatographic method of separation:***

Reverse phase chromatography is selected since all the three drugs are polar in nature.

2. ***Selection of solvent*:**

The drugs exhibited good solubility and stability in methanol. Hence methanol was selected as a solvent.

3. ***Selection of detection wavelength:***

From the overlain spectra 272 was selected as the detection wavelength. (Figure No: 1)

4. ***Optimization of chromatographic conditions:***

Stationary phase	:	Phenomenex C_{18} column
Flow rate	:	1.5 ml per minute
Operation temperature	:	37° C
Selected wavelength	:	231 nm.

a*, **separation using Methanol and water (75 : 25), pH 4.5;***

The retention times are,

Paracetamol : 1.8

Chlorzoxazone : 3.01

Aceclofenac : 4.15

b, separation using Methanol and water (75 : 25), pH 3;

The retention times are,

Paracetamol : 1.85

Chlorzoxazone : 2.93

Aceclofenac : 6.49

c, separation using Acetonitrile and water (60 : 40), pH 3;

The retention times are,

Paracetamol : 1.6

Chlorzoxazone : 2.1

Aceclofenac : 3.08

d, ***separation using Acetonitrile and water (65 : 35), pH 3;***

The retention times are,

Paracetamol : 1.85

Chlorzoxazone : 3.01

Aceclofenac : 3.82

Effect of flow rate:

Peak shapes are better at a flow rate of 1.5 ml per minute than at 1 ml per minute. Hence 1.5ml per minute has been selected for further studies.

Optimized chromatographic conditions:

Stationary phase : Phenomenex C_{18} column

Solvent ratio : Acetonitrile : water (65 : 35)

pH : 3 (with disodium hydrogen ortho phosphate buffer)

Flow rate : 1.5 ml per minute

Operation temperature : 37˚ C

Selected wavelength : 231 nm

Flow rate : 1.5 ml per minute.

Construction of calibration curve:

Taken 2 ml of the standard solutions of aceclofenac, paracetamol and chlorzoxazone in a volumetric flask and made up to 10 ml with mobile phase. Varying quantities of this solution (1, 2, 3, 4 and 5 ml) are diluted with mobile phase in a 10 ml volumetric flask to obtain a solution containing 20-100 µg/ml of aceclofenac, 500 µg/ml of chlorzoxazone and 500 µg/ml of paracetamol. The concentration of the drug was plotted against the area under the curve and shown in Fig 10, 11, and 12. The values are given in table 1, 2 and 3 respectively. The overlain spectrum is given in figure 2.

Market sample analysis:

Preparation of sample solution:

Weighed twenty tablets of the formulation and were crushed to give finely powdered material. Powder equivalent to 25mg of Aceclofenac, 125 mg of paracetamol and 125 mg of chlorzoxazone was

accurately weighed and transferred to a 25ml volumetric flask and were dissolved. It is then diluted to the volume using methanol. It was then sonicated and filtered. Taken 3 ml of the solution and diluted to 10ml in a volumetric flask with methanol. Taken 2ml of the solution and further diluted with mobile phase to 10 ml in a volumetric flask. The solution is then injected into the rheodyne injector. The amount of the drug in each tablet is calculated by using the formula given below.

Amount of drug in each tablet

$$= \frac{\text{Area of sample}}{\text{Area of standard}} \times \text{Conc. of standard} \times \text{Dilution factor} \times \frac{\text{Average weight}}{\text{Weight taken}}$$

The results were furnished in the table 4. The chromatogram is given in Figure 3.

Recovery studies:

It is the measure of the exactness of the analytical method developed accuracy can be reported as percent recovery by the assay of analyte in the sample. The percent recovery values are given in the table 5.

7VALIDATION:

Validation of HPLC method:

1. Specificity:

The conditions of the HPLC methods like percentage of organic solvent in the mobile phase, pH of buffer, flow rate were changed. Although the changes were made, no additional peaks were found but there were some slight changes in Rf and peak shapes.

2. Linearity and range:

Varying quantities of mixed stock solutions was diluted with mobile phase to give concentrations of 20-100 mcg/ml of Aceclofenac and 100-500 mcg/ml of paracetamol and chlorzoxazone. It is then injected into the rheodyne injector.

Calibration curve was plotted using the peak area of Aceclofenac, paracetamol and chlorzoxazone against their corresponding concentrations. The drugs exhibited good linearity as evidenced by the statistical parameters given in the table 1,2, and 3.graphically it is shown in figure 10,11,12.

3. Accuracy:

It is the measure of the exactness of the analytical method developed accuracy can be reported as percent recovery by the assay of

analyte in the sample. The percent recovery values are given in the table 5.

4. Precision;

The precision or reproducibility of the determination under the optimum condition is determined by conducting the determination of the same compound in the freshly prepared stock solution a large number of times. The values are given in the table 7.

5. Limit of detection:

It is the lowest concentration of the analyte in a sample that can be detected. The values are given in the table 8

6. Limit of quantification:

It is the lowest concentration of an analyte in sample that can be determined with acceptable precision and accuracy. The values are given in the table 8.

7. Ruggedness / robustness:

The ruggedness of the analytical method is the degree of reproducibility of the test results obtained by the analysis of the same

samples under a variety of normal test conditions, different analysts, different days etc.

8. System suitability tests:

A system suitability test is the integral part of many analytical procedures. The tests are based on the concepts of equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. It is given in the table 9.

HPLC method using internal standard:

REAGENTS:

STOCK SOLUTION:

Standard stock solutions containing 1mg/ml of aceclofenac, 5 mg/ml of chlorzoxazone, 5 mg/ml of paracetamol and 1 mg/ml are prepared in methanol.

Preparation of mobile phase:

Buffer solution:

Dissolved 3.55 grams of disodium hydrogen ortho phosphate in water and made upto 500 ml with water.

Mobile phase:

Taken 325 ml of acetonitrile and 175 ml of buffer solution and the pH was adjusted to 3 with ortho phosphoric acid. The mobile phase is then filtered through the Ultipor Nylon 66 membrane.

Establishment of R_t:

For determining the R_t values, working standard solutions were prepared taking 2 ml of the standard solutions of aceclofenac, paracetamol and chlorzoxazone in a series of volumetric flask and made up to 10 ml with mobile phase. Diluted 3 ml of these solutions in a series of 10 ml of volumetric flask and added 10 µg/ml of atorvastatin to

obtain 60 µg/ml of aceclofenac and 300 µg/ml of paracetamol and chlorzoxazone. Injected 20 µl of these solutions into the rheodyne injector and the R_t values for the paracetamol, aceclofenac and chlorzoxazone are established as given below

Paracetamol : 1.85
Atorvastatin : 2.42
Chlorzoxazone : 3.01
Aceclofenac : 3.82

Optimization of chromatographic conditions:

1. Selection of chromatographic method of separation:

Reverse phase chromatography is selected since all the four drugs are polar in nature.

2. Selection of solvent:

The drugs exhibited good solubility and stability in methanol.

Hence methanol was selected as a solvent.

3***. Selection of detection wavelength:***

From the overlain spectra 272 nm (figure 4) was selected as the detection wavelength.

4. Optimization of chromatographic conditions:

Separation using Acetonitrile and water (65 : 35), pH 3;

The retention times are,

Paracetamol : 1.85

Atorvastatin : 2.42

Chlorzoxazone : 3.01

Aceclofenac : 3.82

Effect of flow rate:

Peak shapes are better at a flow rate of 1.5 ml per minute than at 1 ml per minute. Hence 1ml per minute has been selected for further studies.

Optimized chromatographic conditions:

Stationary phase : Phenomenex C_{18} column

Solvent ratio : Acetonitrile : water (65 : 35)

pH : 3 (with disodium hydrogen ortho phosphate buffer)

Flow rate : 1.5 ml per minute

Operation temperature : 37˚ C

Selected wavelength : 272 nm

Flow rate : 1.5 ml per minute.

Construction of calibration curve:

Taken 2 ml of the standard solutions of aceclofenac, paracetamol and chlorzoxazone in a volumetric flask and made up to 10 ml with mobile phase. Varying quantities of this solution (1, 2, 3, 4 and 5 ml) containing 12 µg/ml of atorvastatin are diluted with mobile phase in a 10 ml volumetric flask to obtain a solution containing 20-100 µg/ml

of aceclofenac, 500 µg/ml of chlorzoxazone and 500 µg/ml of paracetamol. The concentration of the drug was plotted against the peak area ratio as shown in Fig 13,14, and 15. The values are given in table 10, 11 and 12. Overlaid spectrum is shown in figure 4.

Market sample analysis:

Preparation of sample solution:

Weighed twenty tablets of the formulation and were crushed to give finely powdered material. Powder equivalent to 25mg of Aceclofenac, 125 mg of paracetamol and 125 mg of chlorzoxazone was accurately weighed and transferred to a 25ml volumetric flask with 5 ml of Atorvastatin stock solution diluted to the volume using methanol. It was then sonicated and filtered. Taken 3 ml of the solution and diluted to 10ml in a volumetric flask with methanol. Taken 2ml of the solution and further diluted with mobile phase to 10 ml in a volumetric flask. The solution is then injected into the rheodyne injector. The chromatogram is given in figure 6. The amount of the drug in each tablet is calculated by using the formula given below.

Amount of drug in each tablet

$$= \frac{\text{Peak area ratio of sample}}{\text{Peak area ratio of standard}} \times \text{Conc. of std.} \times \text{dil.factor} \times \frac{\text{Average weight}}{\text{Weight taken}}$$

The results were furnished in the table 13.

Recovery studies:

It is the measure of the exactness of the analytical method developed accuracy can be reported as percent recovery by the assay of analyte in the sample. The percent recovery values are given in the table14.

VALIDATION:

Validation of HPLC method:

1. Specificity:

The conditions of the HPLC methods like percentage of organic solvent in the mobile phase, pH of buffer, flow rate were changed. Although the changes were made, no additional peaks were found but there were some slight changes in Rf and peak shapes.

2. Linearity and range:

Varying quantities of mixed stock solutions was diluted with mobile phase to give concentrations of 20-100 mcg/ml of Aceclofenac and 100-500 mcg/ml of paracetamol and chlorzoxazone. It is then injected into the rheodyne injector.

Calibration curve was plotted using the peak area ratios of aceclofenac, paracetamol and chlorzoxazone against their corresponding concentrations. The drugs exhibit good linearity in the said range as

evidenced by the correlation coefficients given in the table 15. The statistical parameters are given in table 15.

3. Accuracy:

It is the measure of the exactness of the analytical method developed accuracy can be reported as percent recovery by the assay of analyte in the sample. The percent recovery values are given in the table 14.

4**.** ***Precision;***

The precision or reproducibility of the determination under the optimum condition is determined by conducting the determination of the same compound in the freshly prepared stock solution a large number of times. The values are given in the table 16.

5**.** ***Limit of detection:***

It is the lowest concentration of the analyte in a sample that can be detected. The values are given in the table17

6. ***Limit of detection and quantification:***

It is the lowest concentration of an analyte in sample that can be determined with acceptable precision and accuracy. The values are given in the table 17.

7. ***Ruggedness / robustness:***

The ruggedness of the analytical method is the degree of reproducibility of the test results obtained by the analysis of the same samples under a variety of normal test conditions, different analysts, different days etc.

8, System suitability tests:

A system suitability test is the integral part of many analytical procedures. The tests are based on the concepts of equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. It is given in the table 18.

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY:

High performance thin layer chromatography involves the equilibration of a compound between a mobile phase and a thin stationary phase bound to a flat plate. The smaller particle size compared to the thin layer chromatography results in the better resolution.

HPTLC techniques involve the normal phase or reverse phase separation. Normal phase HPTLC is done with precoated unmodified silica gel plates and the reverse phase HPTLC with chemically modified RP-2, RP-8, and RP-18 silica gel plates. In the present work normal phase HPTLC separation was done with precoated unmodified silica gel plates.

Most of the drugs in the formulation can be analyzed by HPTLC method because of the advantages like rapidity, specificity, accuracy and precision. HPTLC method eliminates tedious extraction and isolation procedures.

INSTRUMENTS USED:

CAMAG LINOMAT IV (Schimadzu dual wavelength scanner)

Plate material : HPTLC Silica (merck)

Application mode : CAMAG Sample applicator.

Application chamber : CAMAG twin through glass chamber.

Hamilton syringe – 25 µl

CAMAG TLC Scanner version 3.20

CHEMICALS AND REAGENTS:

Toluene: analytical grade from E-Merck.

Ethyl acetate: analytical grade from E-Merck.

Glacial acetic acid: HPLC grade from E-Merck.

Chloroform: HPLC grade from E-Merck

Methanol: HPLC grade from E-Merck.

Ammonia: analytical grade from qualigens.

MOBILE PHASE:

The mobile phase is prepared by mixing 35 ml of methanol with 10 ml of ethyl acetate and then 1 ml of glacial acetic acid is added to get the required mobile phase.

PREPARATION OF STOCK SOLUTIONS:

Standard stock solutions of Aceclofenac (1mg,/ml), Paracetamol (5mg/ml) and chlorzoxazone (5mg/ml) were prepared in methanol.

ESTABLISHMENT OF R_f VALUES:

For determining the R_f values, working standard solutions were prepared by diluting 3 ml of aceclofenac, paracetamol and chlorzoxazone from the stock solution to a series of 10ml volumetric flasks and making up the volume with methanol. Spotted 15 µl of the solution on the TLC plates and the R_f values for the paracetamol, aceclofenac and chlorzoxazone are established.

The Rf values are

Paracetamol = 0.12
Aceclofenac = 0.29
Chlorzoxazone = 0.72

OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS:

a) Separation using $CHCl_3$+ CH_3OH + NH_3 (48:11:05.):

The Rf values are
Chlorzoxazone - 0.9
Aceclofenac - 0.62
Paracetamol - 0.60

b) Separation using Toluene + ethyl acetate + glacial acetic acid (15+10+0.05):

The Rf values are

Paracetamol - 0.17

Aceclofenac - 0.3

Chlorzoxazone - 0.7

c) Separation using Tolune +ethyl acetete + glacial acetic acid (15 + 10 + 0.2):

The Rf values are

Paracetamol = 0.19

Aceclofenac = 0.30

Chlorzoxazone = 0.72

d) Separation using Toluene +ethyl acetate + glacial acetic acid (17.5 +10+0.5):

The Rf values are

Paracetamol = 0.12

Aceclofenac = 0.29

Chlorzoxazone = 0.72

OPTIMIZED CHROMATOGRAPHIC CONDITION:

Stationary phase : Silicagel Gf 254

Mobile Phase : Toluene + ethyl acetate + glacial acetic acid (17.5 + 10 +0.5)

Lamp : Deuterium

Wavelength : 272 nm

Migration distance : 70 mm

Band with : 3 mm

Distance between the tracks: 10 mm

Rf values :

Paracetamol = 0.12

Aceclofenac = 0.29

Chlorzoxazone = 0.72

CONSTRUCTION OF CALIBRATION CURVE:

Weighed 25mg of Aceclofenac, 125 mg of paracetamol and 125 mg of chlorzoxazone and were dissolved in methanol and made up the volume to 25ml in a volumetric flask. A series of dilution is then made by diluting 1, 2, 3, 4 and 5 ml of the solution in a 10ml standard flask. The concentration range for aceclofenac, paracetamol and chlorzoxazone was 0.1-0.5mg/ml, 0.5-2.5 mg /ml and 0.5-2.5mg /ml respectively. The solution is then spotted (15 µl) on the TLC plates by using automatic application device. The chromatographic plate is then developed in a saturated twin through chamber containing the mobile phase. After development the plates were scanned at 293 nm and the peak areas were measured and given in the Table No 19, 20, and 21. Calibration curve was constructed by plotting concentration against peak

areas as shown in Fig 16,17 and 18. The chromatogram is given in figure 7.

MARKET SAMPLE ANALYSIS:

Weighed twenty tablets of the formulation and were crushed to give finely powdered material. Powder equivalent to 25mg of Aceclofenac, 125 mg of paracetamol and 125 mg of chlorzoxazone was accurately weighed and transferred to a 25ml volumetric flask and were dissolved. It is then diluted to the volume using methanol. It was then sonicated and filtered. Taken 3 ml of the solution and diluted to 10ml in a volumetric flask with methanol. Spotted 15 µl/ml of standard and sample in the precoated TLC plate using automatic application device. The chromatographic plate was developed in a twin through chamber containing the mobile phase. After development the well resolved bands of the drug were scanned at 231 nm. The amount of aceclofenac, paracetamol and chlorzoxazone present in the tablet is calculated by the formula given below.

Amount of drug in each tablet

$$= \frac{\text{Area of sample}}{\text{Area of standard}} \times \text{Conc. of standard} \times \text{Dilution factor} \times \frac{\text{Average weight}}{\text{Weight taken}}$$

The amount present in each tablet is given in the table 22. The chromatogram is given in figure 8.

RECOVERY STUDY:

The accuracy of an analytical method is the test results obtained by the method to the true value. Accuracy may often be expressed as percent recovery by the assay in which a known amount of analyte was added.

It is calculated by the formula.

$$\text{Percentage recovery} = \frac{N\sum xy - (\sum x \sum y)}{N\sum x^2 - (\sum x)^2}$$

X = Amount of drug added in mg/g

N = Total no. of observations

The Percentage Recovery is given in the Table No: 23

VALIDATION:

The developed analytical method was validated for

i) Specificity:

For demonstrating specificity, the conditions of the HPTLC method developed namely the percentage of the organic solvent in the mobile phase, volume etc. was changed and no additional peaks were observed.

ii) ***Linearity and Range:***

The linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration of the analyte in sample within a given range.

The linearity of an analytical method is determined by a mathematical treatment of test results obtained by the analysis of samples with analyte concentration across the claimed range of the method.

It is done usually through the construction of regression line and calculating its slope and intercept which is given in table 24. Alternatively it cane be done graphically by plotting the test results as a function of analyte concentration which is shown in the figure 15, 16 and 17.

iii) Accuracy:

The accuracy of an analytical method is the test results obtained by the method to the true value. Accuracy may often be expressed as percent recovery by the assay in which a known amount of analyte was added.

It is calculated by the formula.

$$\text{Percentage recovery} = \frac{N\sum xy - (\sum x \sum y)}{N\sum x^2 - (\sum x)^2}$$

X = Amount of drug added in mg/g

N = Total no. of observations

The Percentage Recovery is given in the table 23.

iv) Precision;

The precision or reproducibility of the analytical method is determined by assaying a sufficient number of test solutions of the same compound and calculating its standard deviation and relative standard deviation.

The S.D is calculated by the formula

$$S.D = \sqrt{\frac{\sum(X-x)^2}{N-1}}$$

Where $X = \frac{\sum x}{N}$

(X-x) = derivation from mean.

$$C.V = 100 \times \frac{S}{X.} \%$$

The intra day precision is given in the table 25.

v) Limit of Quantification:

It is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. The values are given in table 26.

vi) Limit of Quantification:

It is the lowest concentration of an analyte in a sample detected under the stated experimental conditions. The values are given in table 26

vii) Ruggedness / Robustness:

Ruggedness is a measure of reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst.

viii) System suitability tests:

System suitability tests ensure that the method developed can generate results of acceptable accuracy and precision and values are given in table 27.

DIFFERENTIAL SPECTROSCOPY

Difference spectrophotometric assay is based on the principle in which the measured value of difference absorbance Δ A between two equimolar solutions of the analyte in different chemical forms exhibit different spectral characteristics.

The selectivity and accuracy of spectrometric assay of sample containing absorbing interferents may be improved by the technique of difference spectrophotometry.

Criteria for difference spectroscopy:

i. Reproducible changes may be induced in the spectrum of the analyte by the addition of one (or) more reagents.

ii. The absorbance of the interfering substance is not changed by the reagents.

Difference spectrophotometry is simplest and most commonly employed technique for altering the spectral properties of the analyte. It is done by adjusting the pH by means of aqueous solutions of acid, alkali (or) buffers.

The UV –visible absorption spectra of the substance containing lonisable functional groups will depend upon the state of ionization of

the functional group and consequently the pH of the solution. Examples of such functional groups include phenols, aromatic carboxylic acids and amines

If the individual absorbance, A_{alk} and A_{acid} are proportional to the concentration of the analyte and pathlength , then ΔA also obeys the Beer-Lambert Law and a modified equation may be derived.

$$\Delta A = \Delta abc$$

ΔA is the difference absorptivity of the substance at the wave length of measurement.

INSTRUMENT EMPLOYED:

Schimadzu UV-1650 PC UV/VIS Spectrophotometer.

Mettler AB54 balance

Chemicals and reagents:

Methanol analytical grade (E-Merck)

Sodium hydroxide 0.1M

Dissolved 2g of sodium hydroxide in distilled water and made up to 500 ml to obtain 0.1M sodium hydroxide.

Hydrochloric acid 0.1M:

Mixed 1.825 ml of 1M hydrochloric acid in distilled water and made upto 500 ml.

PREPARATION OF STANDARD STOCK SOLUTION:

Dissolved 30mg of aceclofenac in methanol and made up the volume to 25 ml in a volumetric flask to obtain a standard stock solution containing 1.2 mg/ml of aceclofenac.

Experiment:

Establishment of absorbtion maximum:

The standard stock solution was suitably diluted to give the concentration of 0.45 µg/ml. The dilutions are made with 0.1M hydrochloric acid and 0.1M sodium hydroxide in two separate volumetric flasks. The solutions are then scanned in the UV region (200-400nm) keeping the corresponding reagent blank. The λmax for acidic (0.1M hydrochloric acid) and alkaline (0.1M sodium hydroxide) solutions were observed at 272 nm and 218 nm respectively.It is shown in the figure 9.

Construction of calibration graph:

The standard stock solution was suitably diluted to give the concentration of 0.96 µg/ml to 96 µg/ml of aceclofenac. The dilutions

were made separately using 0.1M sodium hydroxide and 0.1M hydrochloric acid. The absorbances were then measured 272 nm for both acidic and alkaline solutions of the drug and the difference absorbance (ΔA) is plotted against the concentration. (Figure 19) and the data were given in shown in the table 28.

Market sample analysis:

The average weight of twenty tablets were determined and ground to a fine powder. An accurately weighed sample equivalent to 30 mg of aceclofenac was transferred to a 25 ml volumetric flask. It was shaken with 20 ml of methanol, sonicated and made up to the volume with methanol. The solution was then filtered through the Whatmann filter No.1. The solution was then diluted with 0.1M hydrochloric acid and 0.1M sodium hydroxide in two separate volumetric flask to obtain the concentration of 0.54 μg/ml.

Amount of aceclofenac present in each tablet is calculated using the formula mentioned below,

Amount of drug in each tablet

$$= \frac{\Delta A \text{ of sample}}{\Delta A \text{ of standard}} \times \text{Conc. of standard} \times \text{Dilution factor} \times \frac{\text{Average weight}}{\text{Weight taken}}$$

The results were given in table 29.

Recovery study:

To ensure the accuracy and reproducibility of the above method, recovery studies were carried out by adding a known quantity of standard drug with the pre analyzed sample formulation and the percentage recovery was calculated. The values are given in the table 30.

Validation:

Validation of differential spectroscopic method:

1. Specificity:

The conditions of the differential spectroscopic methods like pH of hydrochloric acid and sodium hydroxide were changed. Although the changes were made, there is no change in the λmax.

2. Linearity and range:

Varying quantities of stock solutions was diluted with methanol to give concentrations of 9.6 to 96 µg/ml of aceclofenac. The absorption was then measured in the UV spectrophotometer. Table 33 shows the statistical data obtained. The calibration graph was found to be linear in the concentration range of 9.6 to 96 µg/ml with the correlation coefficient of 0.9997. The results indicated good linear relationship between peak area and concentration.

3. Accuracy:

It is the measure of the exactness of the analytical method developed accuracy can be reported as percent recovery by the assay of analyte in the sample. The percent recovery values are given in the table 30.

4. Precision;

The precision or reproducibility of the determination under the optimum condition is determined by conducting the determination of the same compound in the freshly prepared stock solution a large number of times. The values are given in the table. The results in table 31 indicate that the method is highly precise.

5. Limit of detection:

It is the lowest concentration of the analyte in a sample that can be detected. The values are given in the table 32.

6. Limit of quantification:

It is the lowest concentration of an analyte in sample that can be determined with acceptable precision and accuracy. The values are given in the table 32.

7. Ruggedness / robustness:

The ruggedness of the analytical method is the degree of reproducibility of the test results obtained by the analysis of the same samples under a variety of normal test conditions, different analysts, different days etc

Figure No: 1

Overlaid spectrum of aceclofenac, paracetamol and chlorzoxazone.

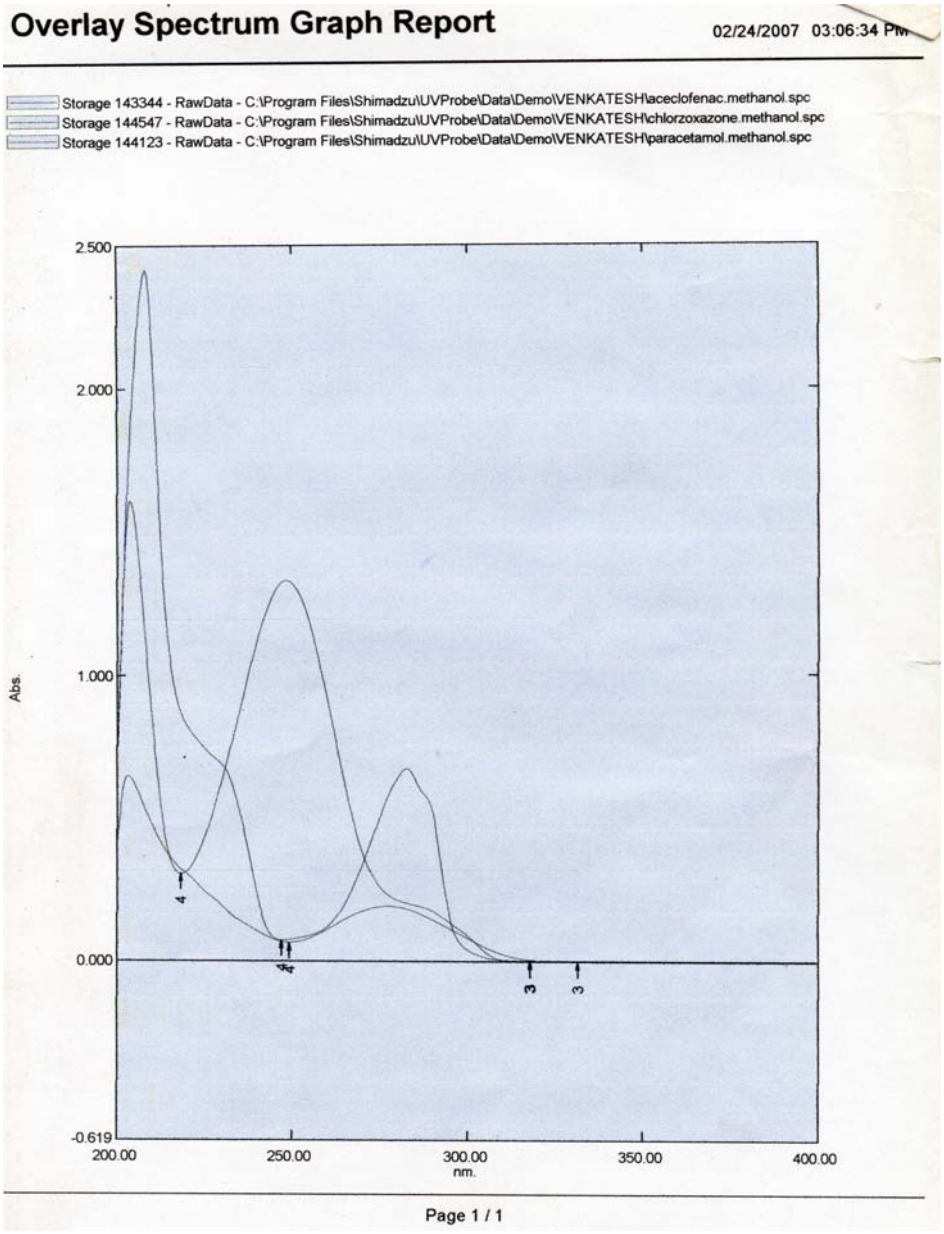


Figure No: 4

Overlaid spectrum of aceclofenac, paracetamol, chlorzoxazone and atorvastatin.

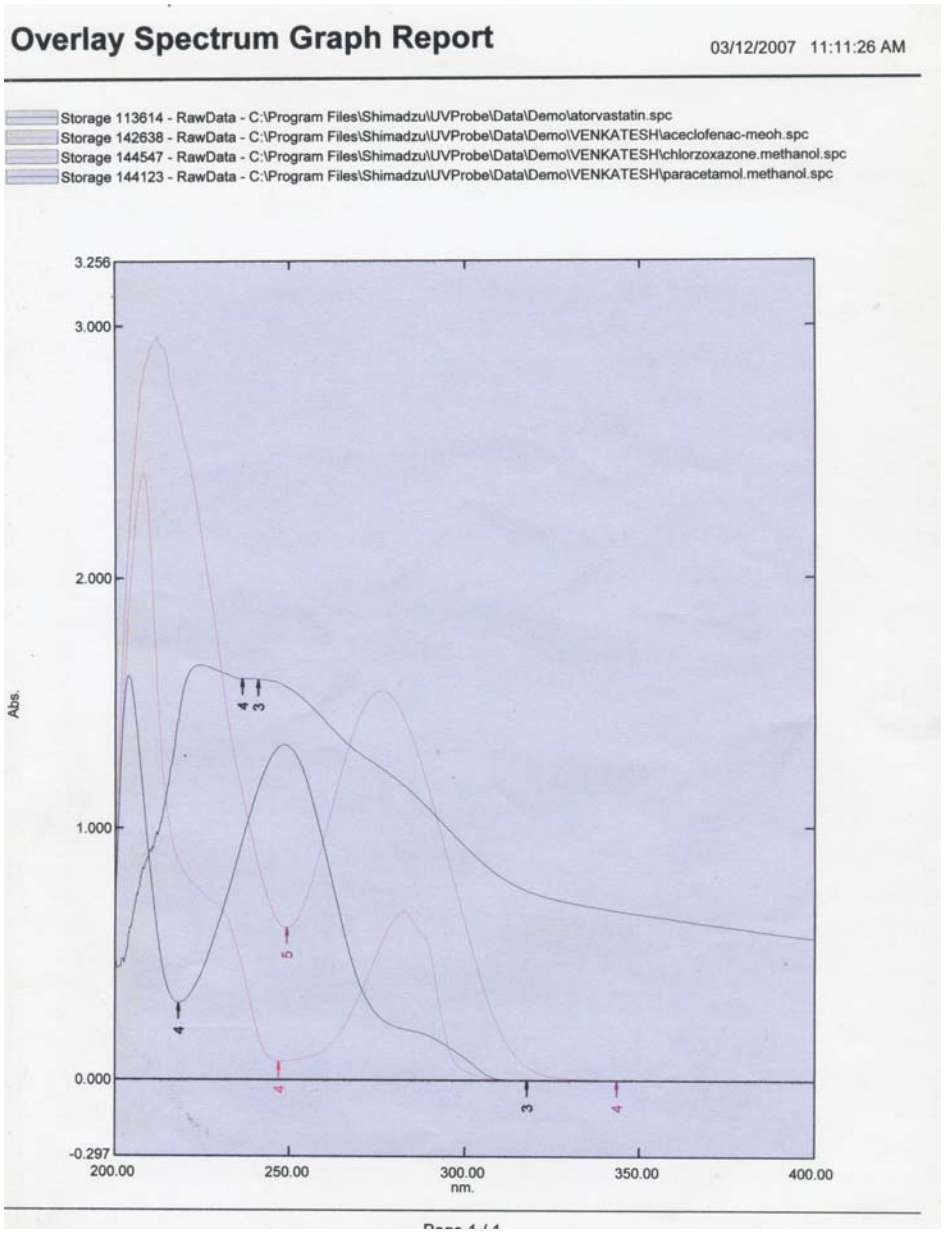


Figure No: 16

Calibration graph of aceclofenac (HPTLC)

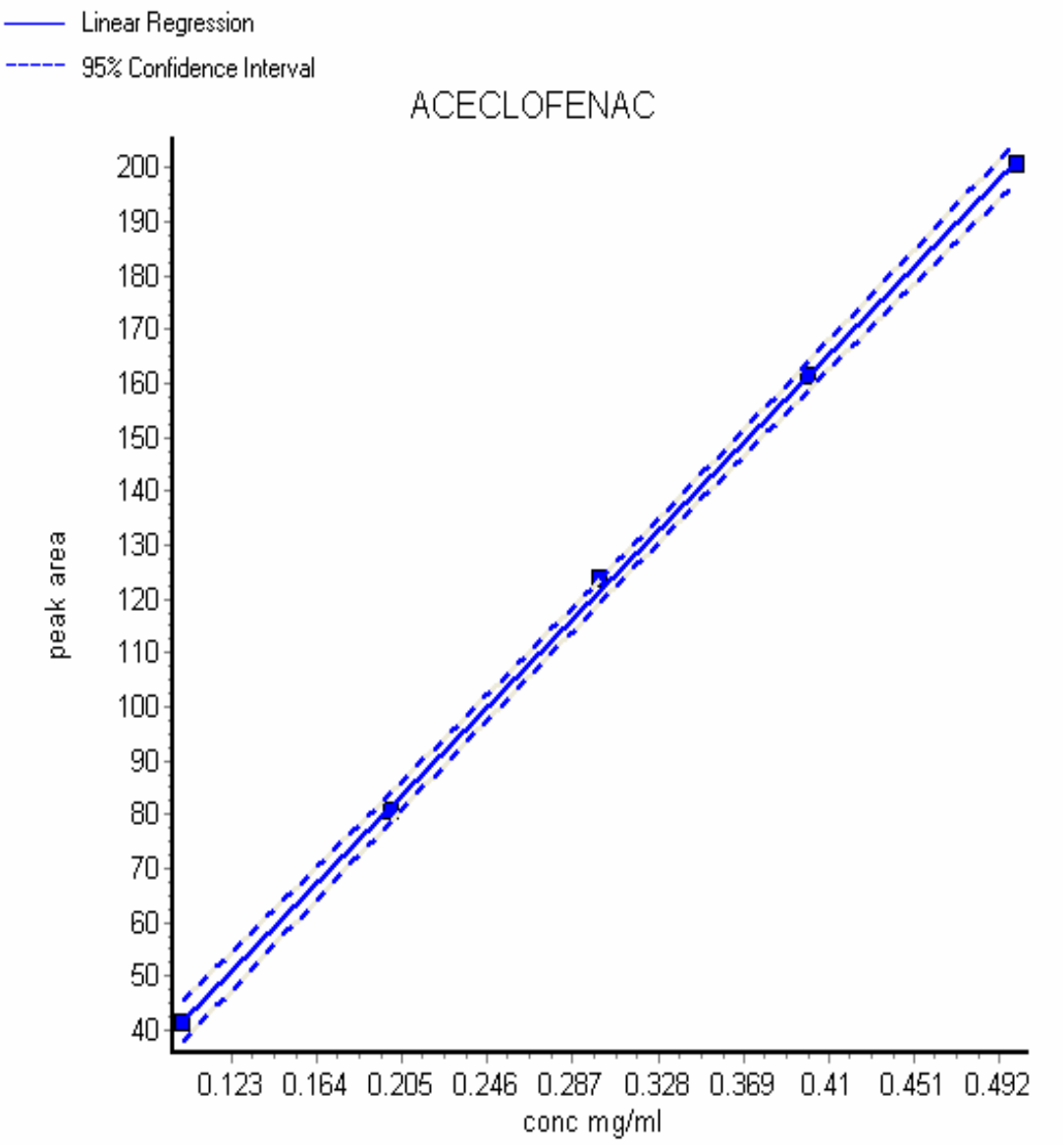


Figure No: 17

Calibration graph of paracetamol (HPTLC)

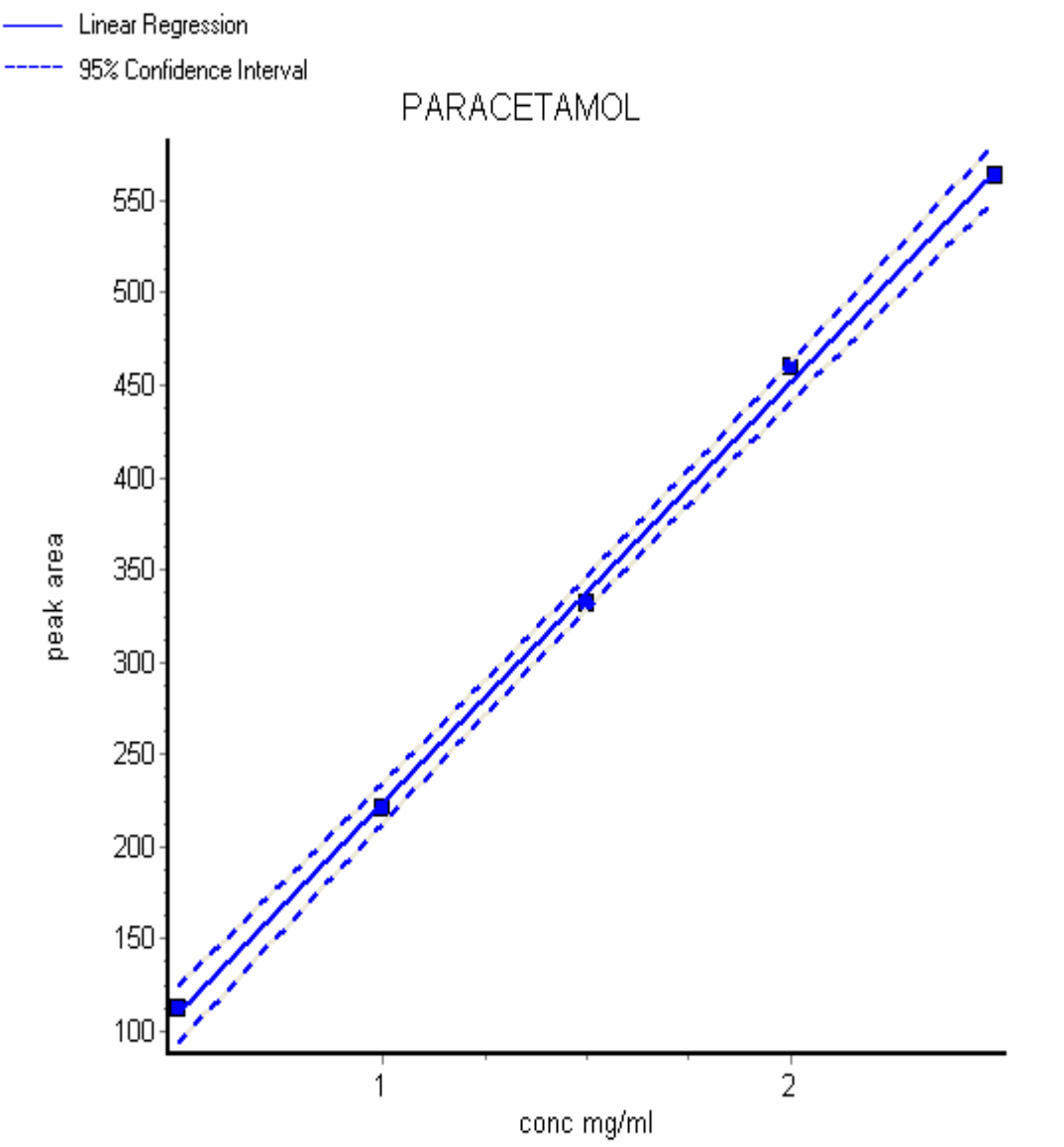


Figure No: 18

Calibration graph of chlorzoxazone (HPTLC)

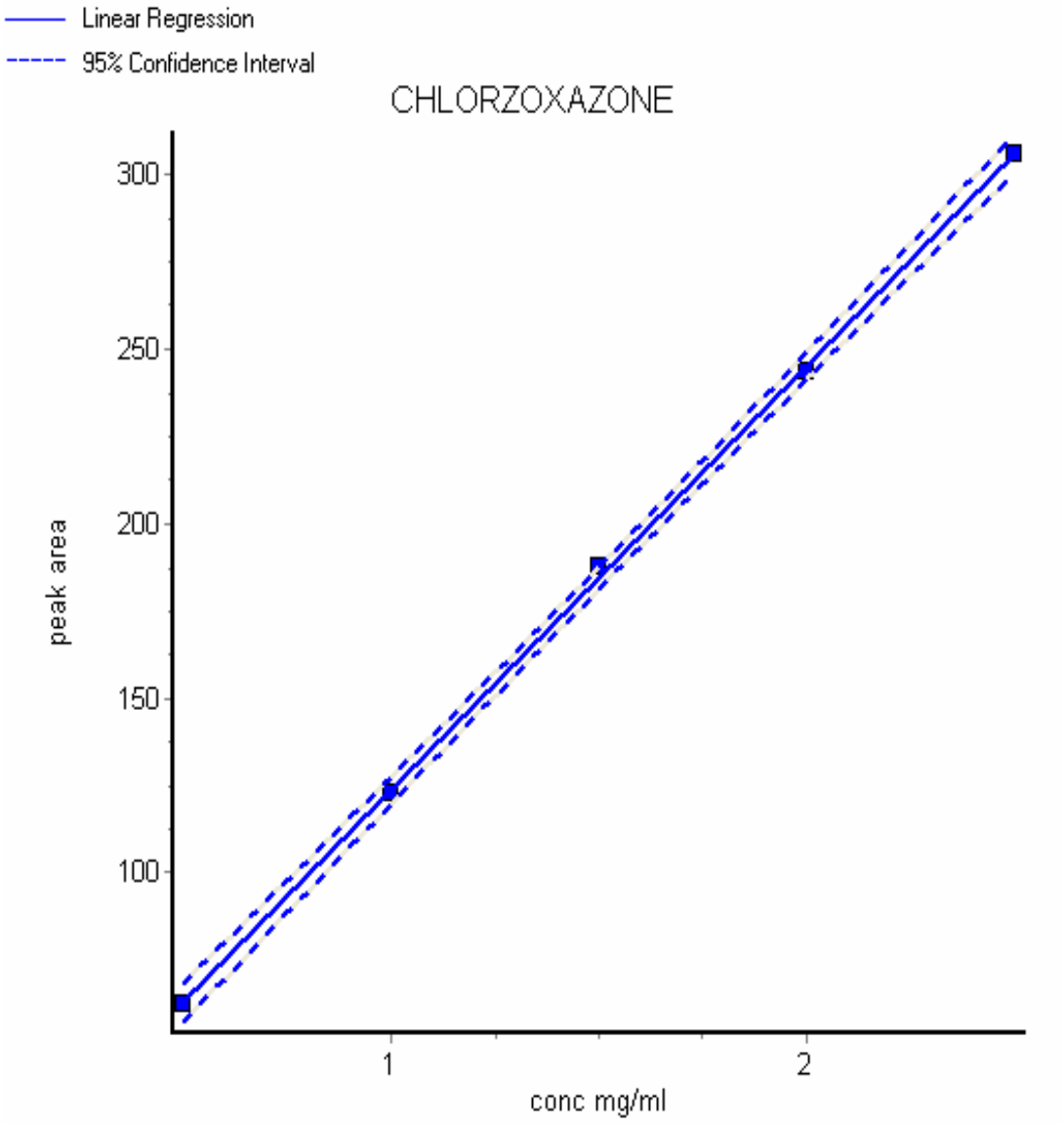


Figure No: 10

Calibration graph of aceclofenac (HPLC)

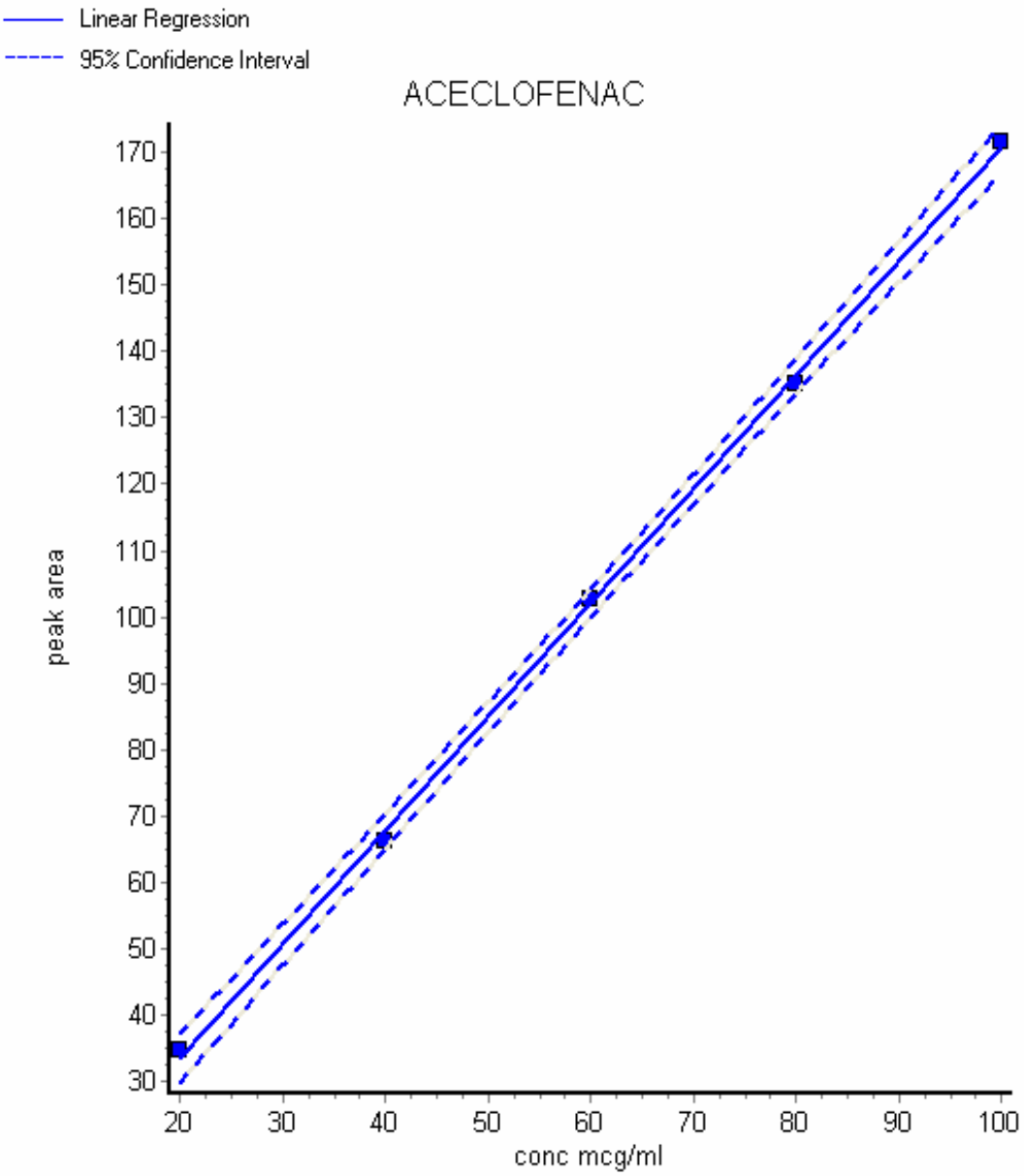


Figure No: 12

Calibration graph of chlorzoxazone (HPLC)

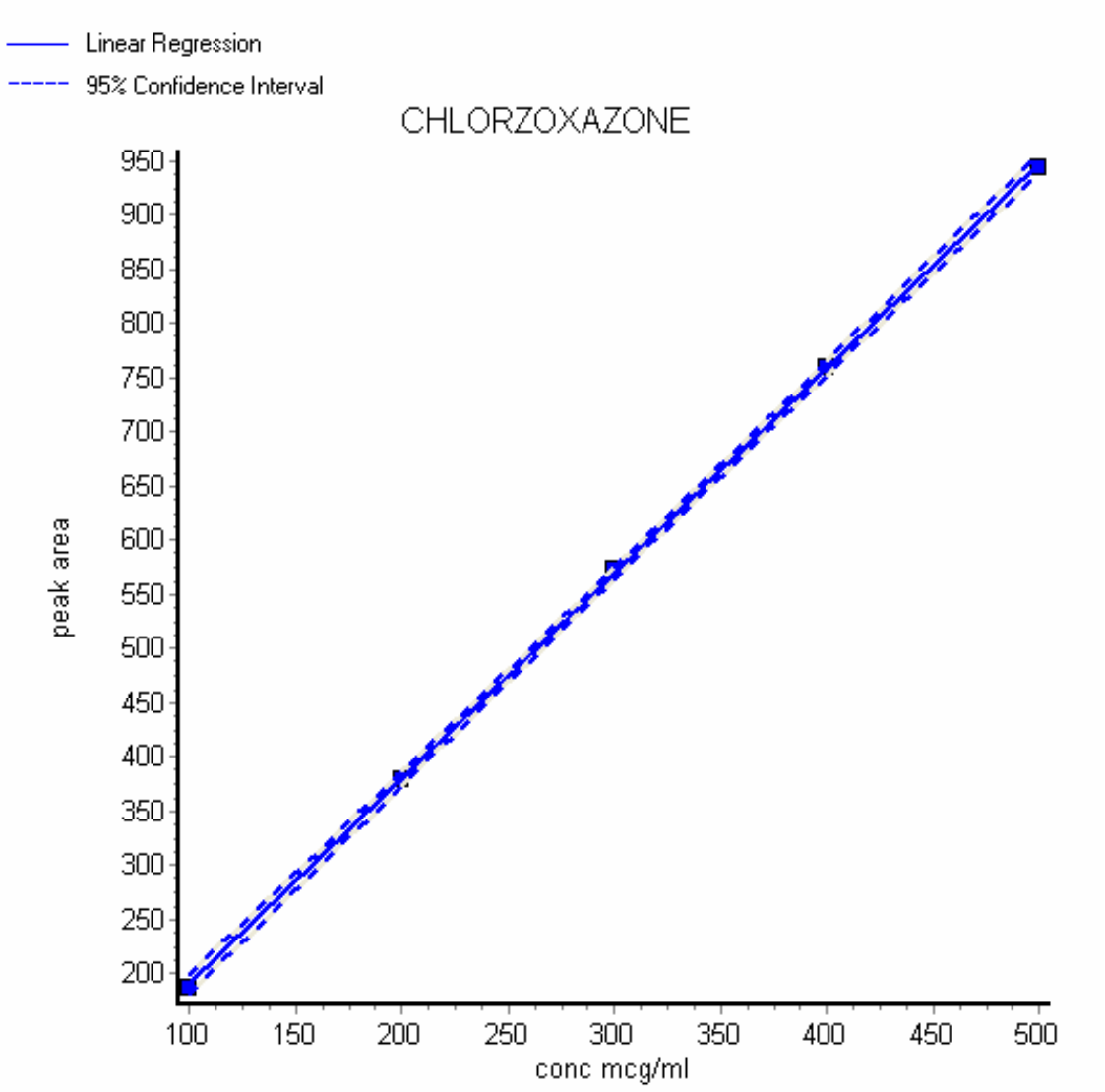


Figure No: 11

Calibration graph of paracetamol (HPLC)

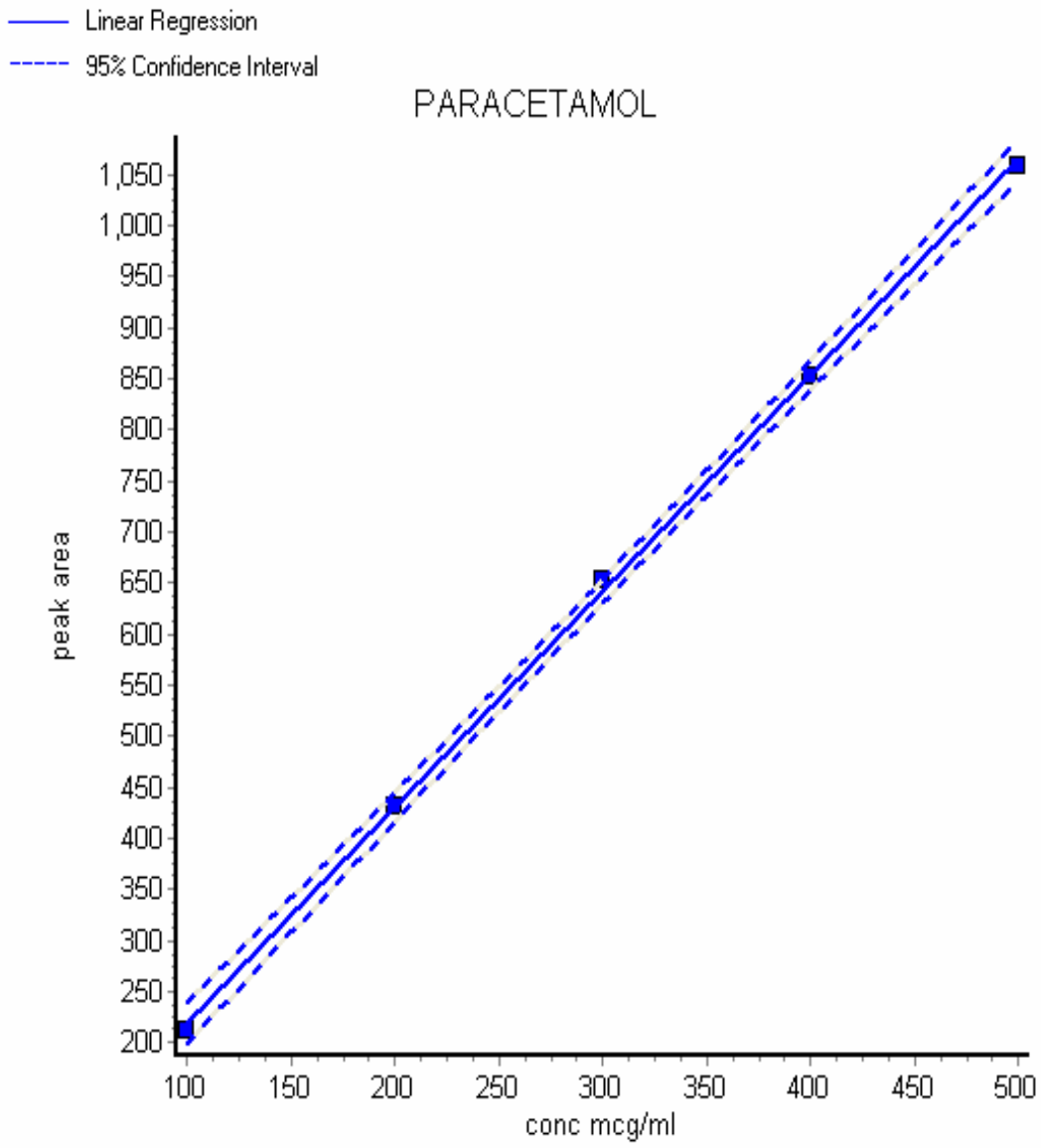


Figure No:13

Calibration graph of aceclofenac (HPLC with internal standard)

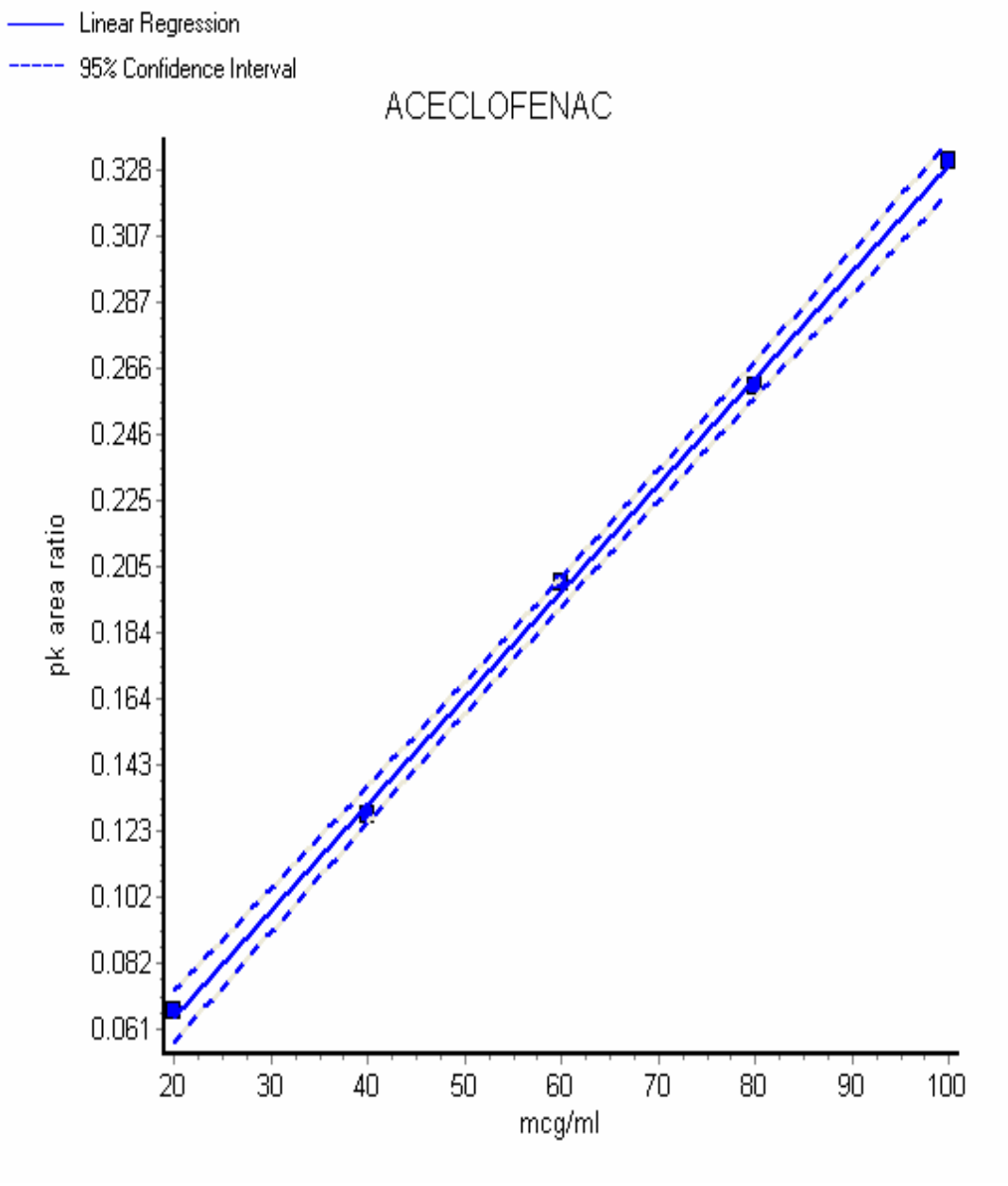


Figure No: 14

Calibration graph of paracetamol (HPLC with internal standard)

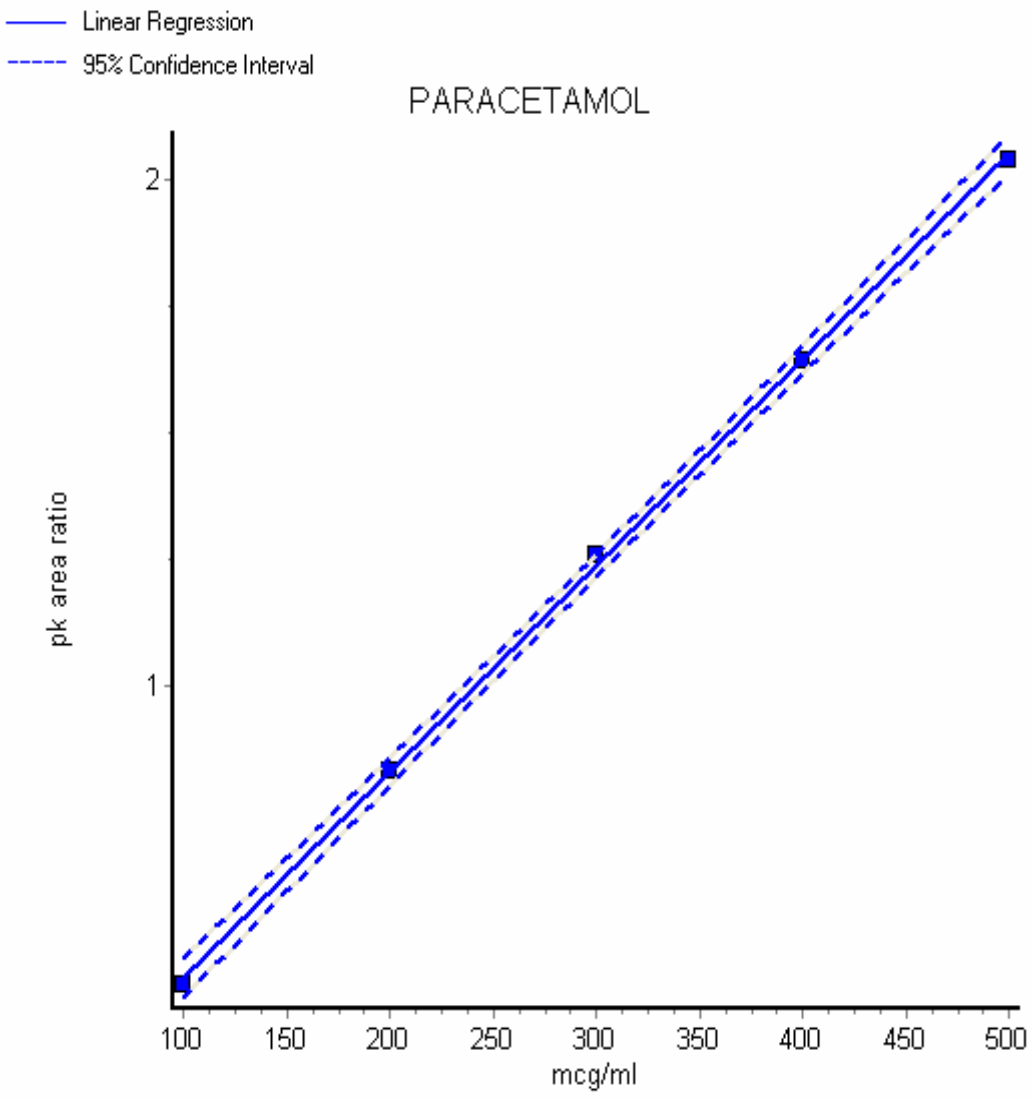


Figure No: 15

Calibration graph of chlorzoxazone (HPLC with internal standard)

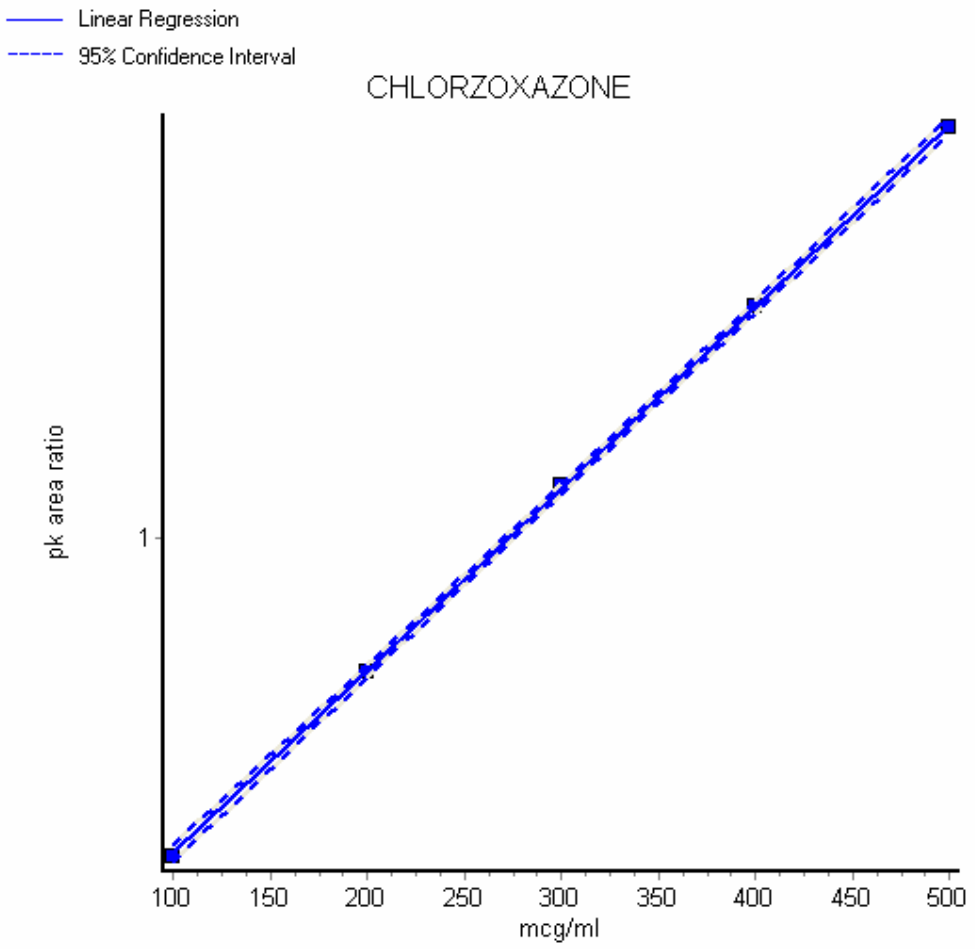


Figure No: 19

Calibration graph of aceclofenac (difference spectroscopy)

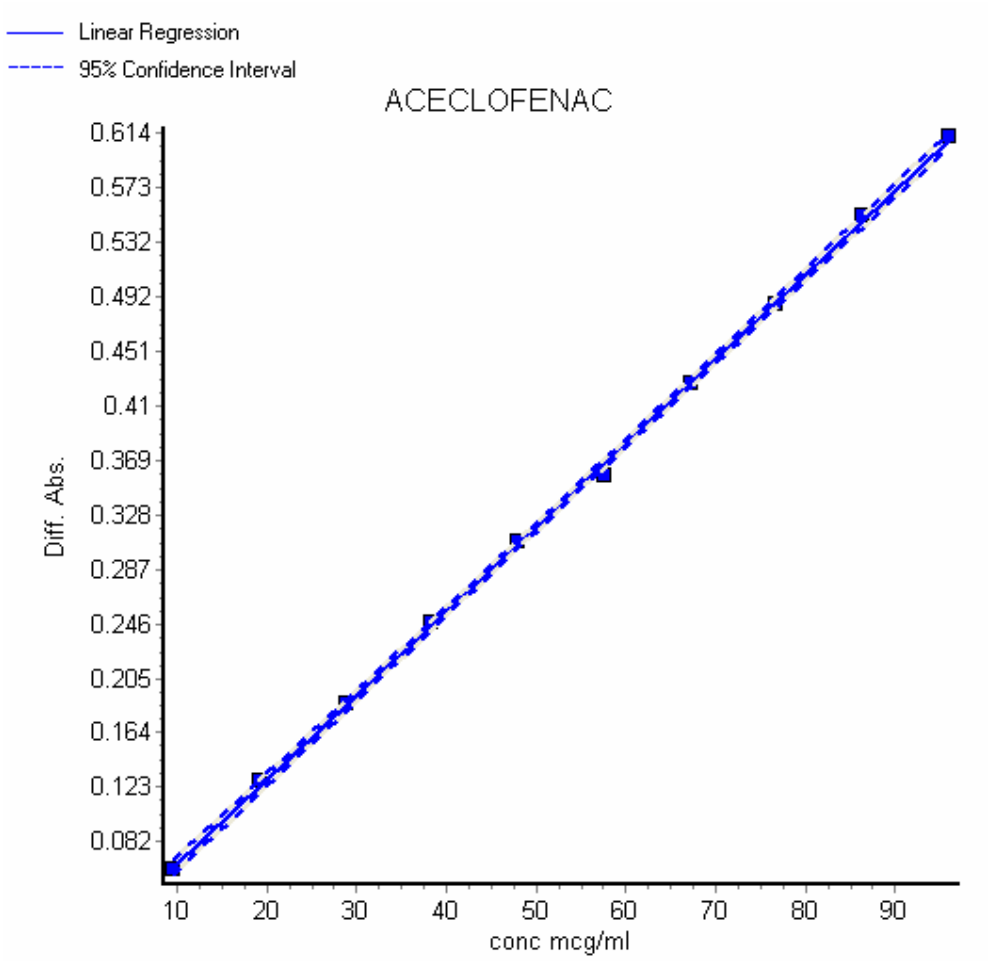


Figure No: 6

Sample chromatogram of aceclofenac, paracetamol and chlorzoxazone with internal standard Atorvastatin.

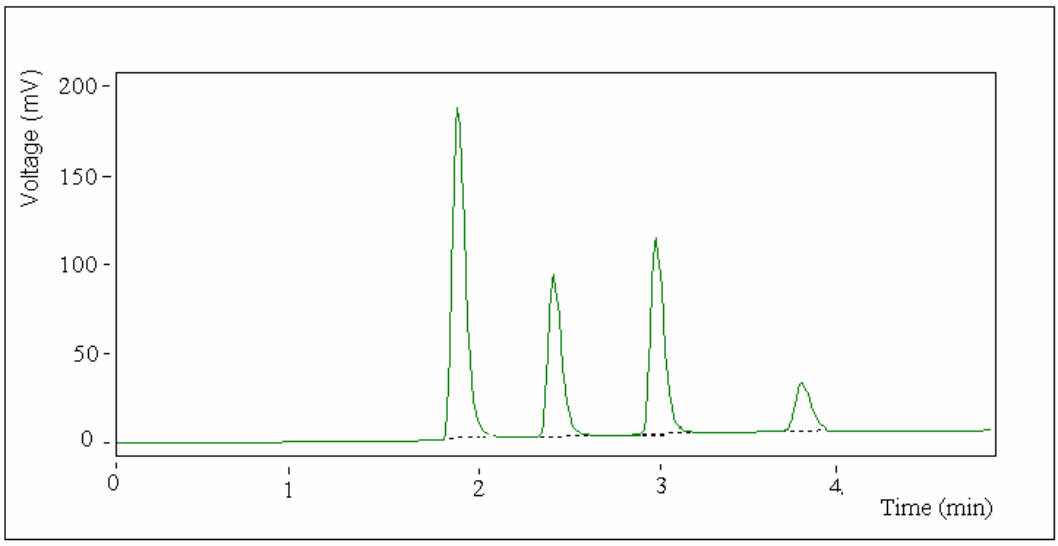


Figure No: 8

Sample chromatogram of aceclofenac, paracetamol and chlorzoxazone (HPTLC)

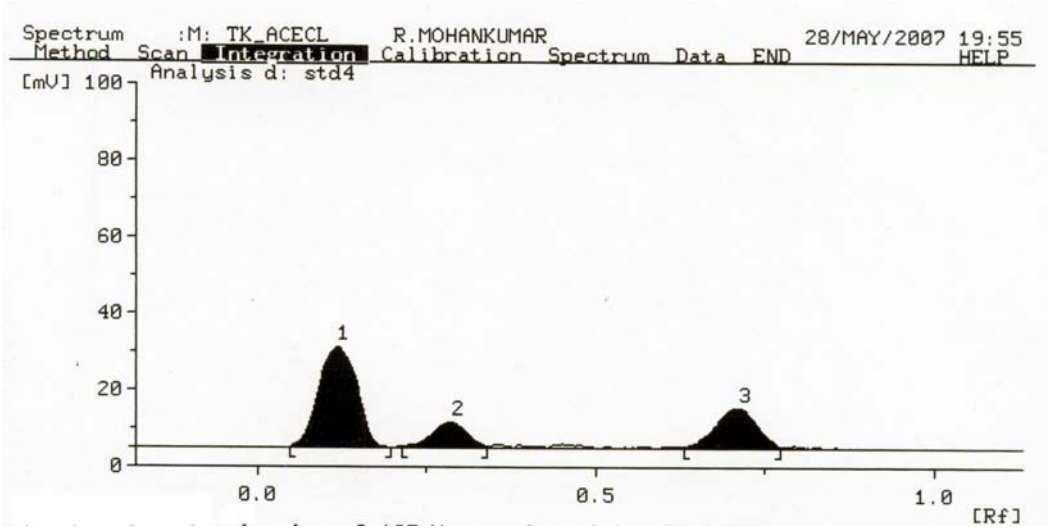


Figure No: 2

Standard chromatogram of aceclofenac, paracetamol and chlorzoxazone

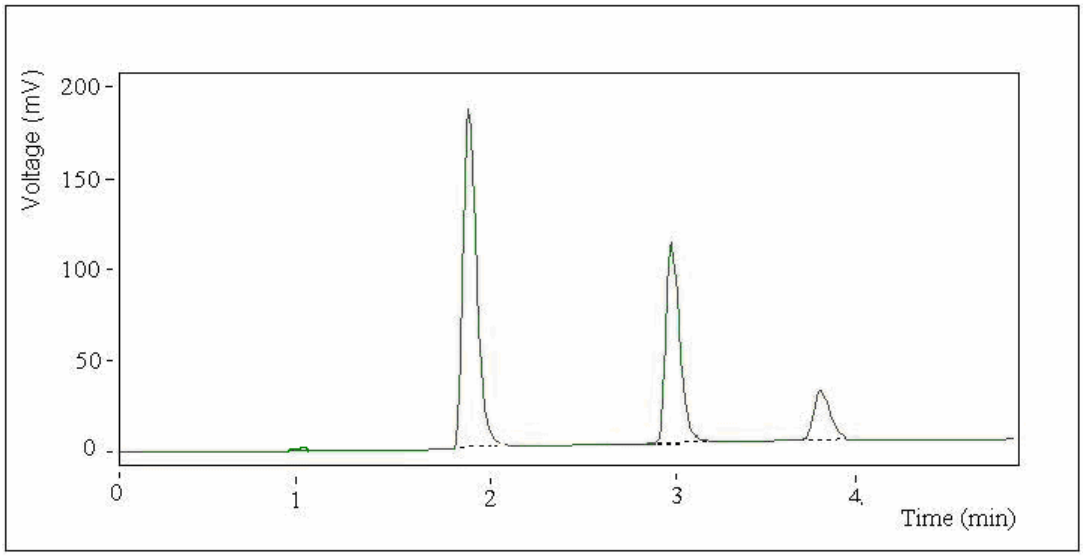


Figure No: 7

Standard chromatogram of aceclofenac, paracetamol and chlorzoxazone (HPTLC)

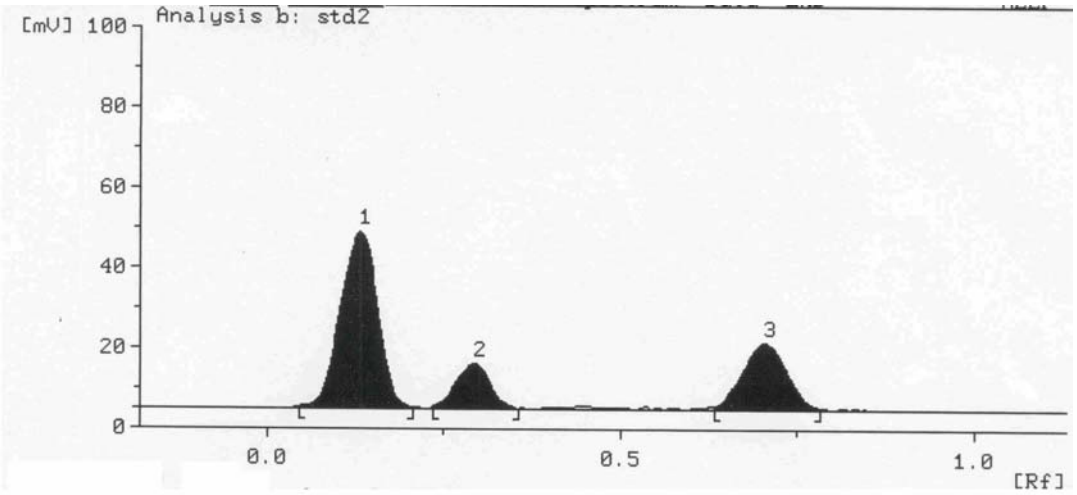


Figure No: 5

Standard chromatogram (overlaid) of aceclofenac, paracetamol and chlorzoxazone with internal standard Atorvastatin (HPLC)

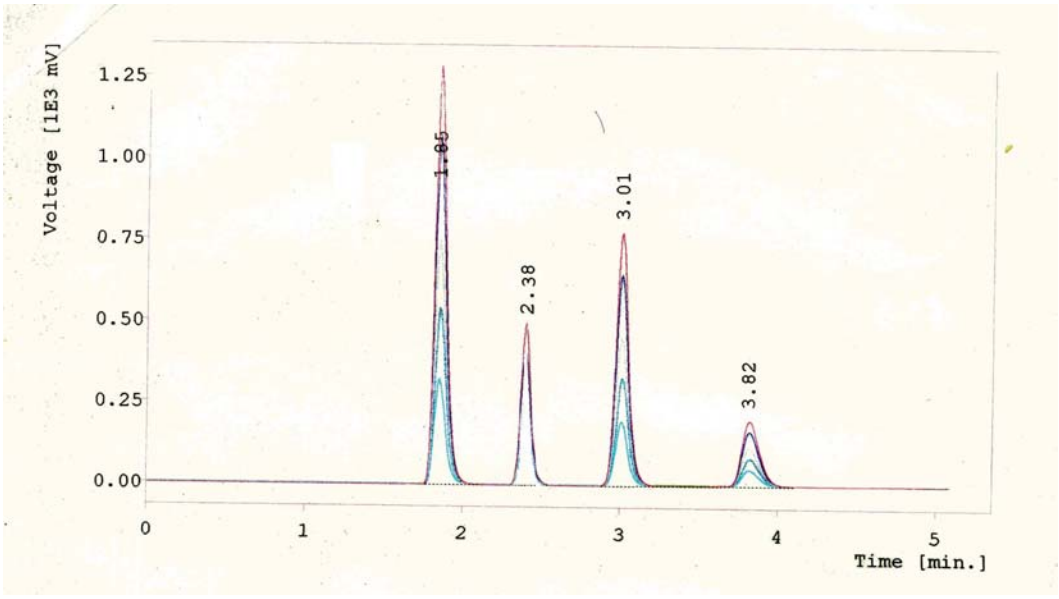


Figure No: 3

Sample chromatogram of aceclofenac, paracetamol and chlorzoxazone

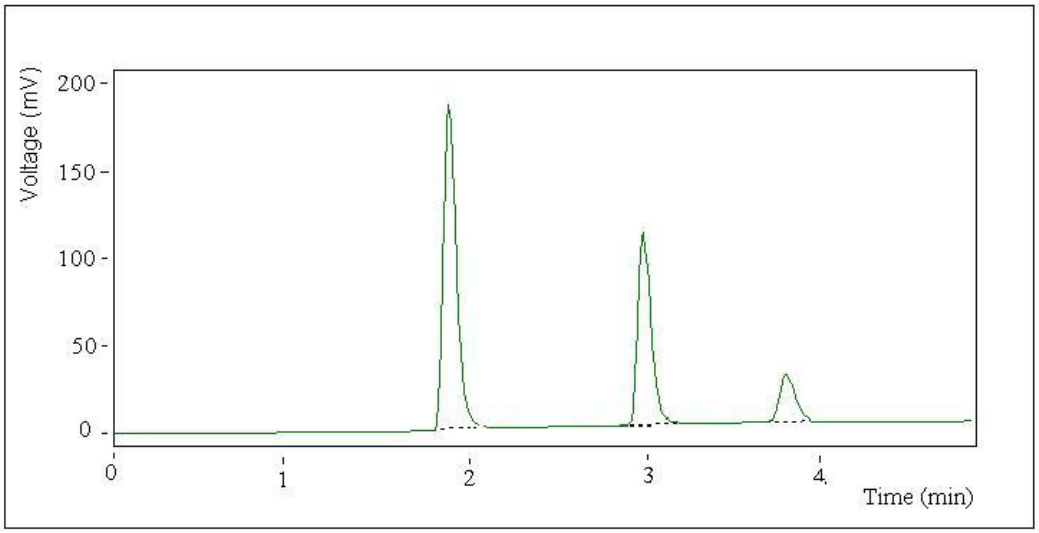


Figure No: 9

Over laid spectra of Aceclofenac in Hcl & NaoH

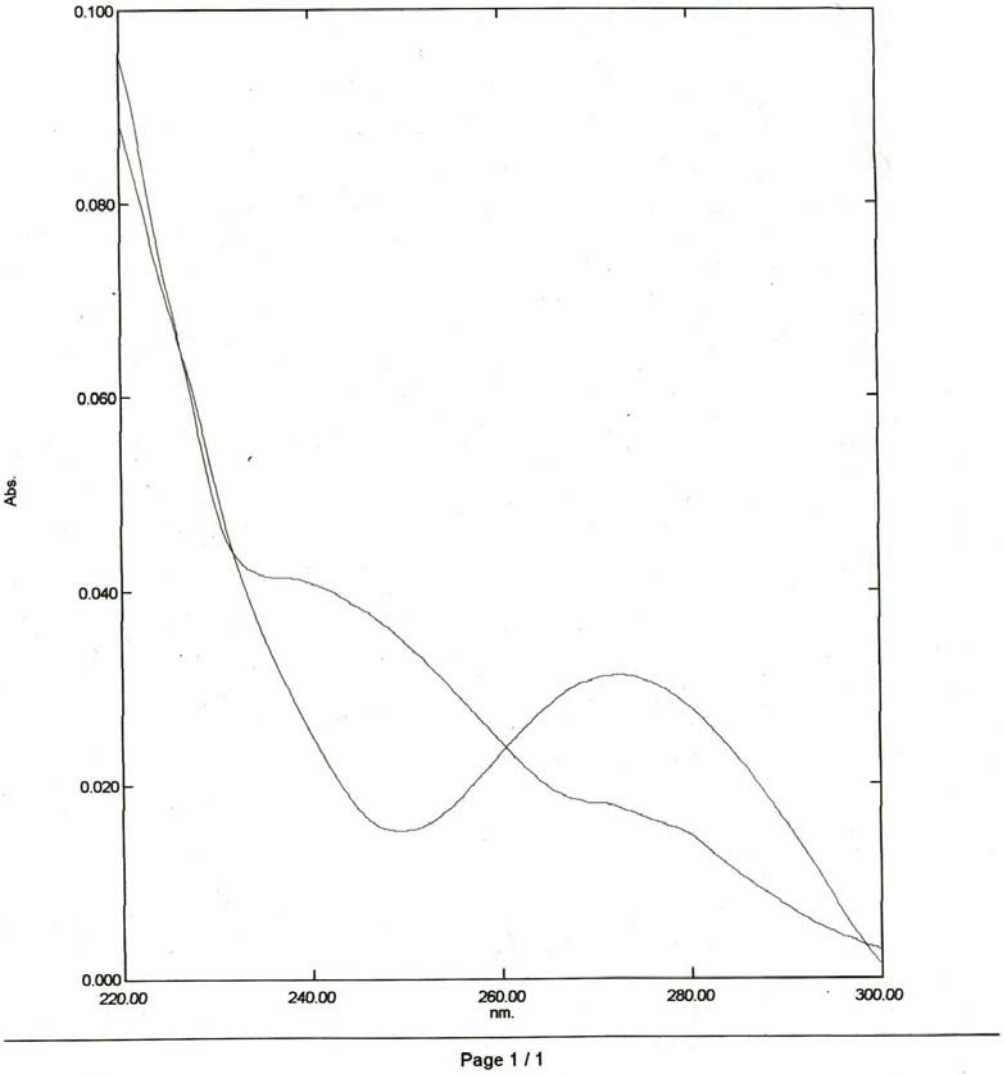


TABLE NO: 1.

Linearity and range of aceclofenac

S.No.	Concentration of aceclofenac in µg/ml	Peak area
1	20	34.6821
2	40	66.1284
3	60	105.3279
4	80	135.0819
5	100	171.4160

TABLE NO : 2

Linearity and range of chlorzoxazone

S.No.	Concentration chlorzoxazone in µg/ml	Peak area
1	100	187.8214
2	200	379.9018
3	300	573.5491
4	400	760.1906
5	500	944.3217

TABLE NO: 3

Linearity and range of paracetamol

S.No.	Concentration paracetamol in µg/ml	Peak area
1	100	212.9615
2	200	430.6160
3	300	653.2281
4	400	853.1782
5	500	1058.6270

TABLE NO: 19.

Linearity and range of aceclofenac (HPTLC)

S.No.	Concentration aceclofenac in mg/ml	Peak area
1	0.1	41.3
2	0.2	80.5
3	0.3	126.7
4	0.4	161.3
5	0.5	200.4

TABLE NO: 20

Linearity and range of paracetamol (HPTLC)

S.No.	Concentration paracetamol in mg/ml	Peak area
1	0.5	111.3
2	1.0	220.8
3	1.5	331.6
4	2.0	460.1
5	2.5	563.9

TABLE NO: 21

Linearity and range of chlorzoxazone (HPTLC)

S.No.	Concentration chlorzoxazone in mg/ml	Peak area
1	0.5	62.2
2	1.0	122.5
3	1.5	187.7
4	2.0	243.6
5	2.5	305.9

TABLE NO: 22

Estimation of aceclofenac, paracetamol and chlorzoxazone (HPTLC)

S.No	Name of the drug	Label claim (mg/tablet)	Amount found (mg/tablet)	standard deviation	Relative standard deviation
1	Aceclofenac	100	102.1286	0.537229	0.52820
2		100	101.8918		
3		100	101.1025		
1	Paracetamol	500	501.9841	0.679907	0.13526
2		500	502.702		
3		500	503.3051		
1	chlorzoxazone	500	495.9923	0.704765	0.14201
2		500	495.7259		
3		500	497.0578		

TABLE NO : 23

Recovery of aceclofenac, paracetamol and chlorzoxazone (HPTLC)

S.No	Drug	Amount of drug in sample (mg)	Amount of drug added (mg)	Amount Recovered (mg)	Percent Recovery
1	Aceclofenac	102.1286	10	9.8655	98.6587
2		101.8918	10	9.9445	99.4451
3		101.1025	10	10.0234	100.2344
1	Paracetamol	501.9481	50	49.6067	99.2135
2		502.702	50	49.7575	99.5150
3		503.3051	50	49.9083	99.8166
1	Chlorzoxazone	495.992	50	49.5459	99.0919
2		495.7529	50	50.0787	100.1574
3		497.0578	50	49.5459	99.0919

TABLE No : 24

Statistical parameters in HPTLC

S.No	Parameter	Aceclofenac	Paracetamol	Chlorzoxazone
1	Regression equation (y)	349.7 x -0.009	228.62 x – 5.530	121.7 x - 0.01504
2	Slope (m)	349.7	228.62	121.7
3	Intercept (b)	-0.009	-5.530	-0.01504
4	Correlation coefficient(r)	0.9979	0.9991	0.9996

TABLE NO : 25

Precision data for aceclofenac, paracetamol and chlorzoxazone (HPTLC)

S.No	Drug	Conc. of the drug in mg/ml	Peak area	Standard deviation	C.V.
1	Aceclofenac	0.3	125.4 125.8 126.5 126.1 126.7 127.6 127.5 127.6	0.8569	0.6769
2	Paracetamol	1.5	330.7 330.8 331.4 330.8 331.6 331.7 332.4 333.2	0.8730	0.2632
3	Chlorzoxazone	1.5	186.3 187.4 187.2 187.6 186.9 188.2 188.9 188.1	0.8172	0.4356

TABLE NO : 26

LOD and LOQ (HPTLC)

S.No	Parameter	Aceclofenac	Paracetamol	Chlorzoxazone
1	LOD	4µg/ml	8 µg/ml	17 µg/ml
2	LOQ	15 µg/ml	29 µg/ml	57µg/ml

TABLE No : 27

System Suitability Parameters (HPTLC)

S.No	Parameter	Paracetamol	aceclofenac	chlorzoxazone
1	R_f	.12	.29	.72
2	Asymmetry factor	1.07	1.02	1.12
3	Theoretical plates/meter	962	1956	4365
4	Linearity	0.5-2.5mg/ml	0.1-0.5mg/ml	0.5-2.5mg/ml

TABLE No : 28

S.No	Concentration of Aceclofenac in µg/ml	Absorbance
1	9.6	0.061
2	19.2	0.127
3	28.8	0.186
4	38.4	0.246
5	48.0	0.307
6	57.6	0.357
7	67.2	0.426
8	76.8	0.486
9	86.4	0.553
10	96.0	0.612

Linearity of Aceclofenac (Differential Spectormetry)

TABLE No: 29

Estimation of Aceclofenac by UV spectrometry (Diferential Spectroscopy)

S.No	Name of the drug	Label claim (mg/tablet)	Amount found (mg/tablet)	standard deviation	Relative standard deviation
1	Aceclofenac	100	101.3036	0.3257	0.3215
2		100	101.6294		
3		100	100.9779		

TABLE - 30

Recovery of Aceclofenac (differential spectrometry)

S.No	Drug	Amount of drug in sample (mg)	Amount of drug added (mg)	Amount Recovered (mg)	Percent Recovery
1	Aceclofenac	101.3036	10	10.0978	100.9780
2		100.9779	10	9.7720	97.7206

TABLE - 31

Precision data for Aceclofenac

S.No	Drug	Conc. of the drug in µg/ml	absorbance	Standard deviation	C.V.
1	Aceclofenac	48.7	0.311 0.312 0.312 0.309 0.313 0.315 0.313 0.312	.0017	.5532

Table - 32

LOD and LOQ (difference spectrometry)

S.No	Parameter	Aceclofenac
1	LOD	6 ng/ml
2	LOQ	20 ng/ml

Table - 5

Recovery of Aceclofenac, Chlorzoxazone & Paracetomal (HPLC)

S.No	Drug	Amount of drug in sample (mg)	Amount of drug added (mg)	Amount Recovered (mg)	Percent Recovery
1	Aceclofenac	101.0852	10	9.9537	99.537
2		100.3345	10	10.09408	100.9408
3		101.335	10	9.9013	99.01351
1	Paracetamol	502.3227	50	50.0827	100.1654
2		501.1098	50	50.7578	101.5157
3		501.1222	50	50.1470	100.294
1	Chlorzoxazone	496.1929	50	50.0765	100.153
2		496.6073	50	49.8977	99.7954
3		498.3508	50	50.1026	100.2053

TABLE - 33

Statistical Parameters for Aceclofenac

S.No	Parameter	Aceclofenac
1	Regression equation (y)	157.83 x – 0.2492
2	Slope (m)	157.83
3	Intercept (b)	-0.2492
4	Correlation coefficient(r)	0.9997

TABLE NO: 4

Estimation of aceclofenac, paracetamol and chlorzoxazone (HPLC)

S.No	Name of the drug	Label claim (mg/tablet)	Amount found (mg/tablet)	standard deviation	Relative standard deviation
1	Aceclofenac	100	101.0852	0.528793	0.52394
2		100	100.3345		
3		100	101.335		
1	Paracetamol	500	502.24	0.648932	0.12940
2		500	501.1098		
3		500	501.1222		
1	chlorzoxazone	500	496.1929	0.656455	0.13214
2		500	496.6073		
3		500	497.479		

TABLE No: 6

Statistical parameters in HPLC

S.No	Parameter	Aceclofenac	Paracetamol	Chlorzoxazone
1	Regression equation (y)	1.712 x + 0.1163	2.114 x – 1.871	1.894 x - 0.3895
2	Slope (m)	1.712	2.114	1.894
3	Intercept (b)	0.1163	-1.871	-0.3895
4	Correlation coefficient(r)	0.993	0.9999	0.9999

Table No: 7

Precision data for aceclofenac, paracetamol and chlorzoxazone (HPLC)

S.No	Drug	Conc. Of the drug in µg/ml	Peak area	Standard deviation	C.V.
1	Aceclofenac	60	104.9736 104.67 105.0826 104.3405 105.5732 105.9858 105.6822 106.3153	0.67474	0.6406
2	Paracetamol	300	634.2738 634.5702 634.9828 634.2407 635.4734 635.886 636.0824 636.2155	0.8084	0.1272
3	Chlorzoxazone	300	573.1948 572.5612 573.3038 572.5617 573.7944 574.537 573.9034 574.5365	0.78131	0.1362

Table No: 8

LOD and LOQ (HPLC)

S.No	Parameter	Aceclofenac	Paracetamol	Chlorzoxazone
1	LOD	0.9 µg/ml	0.9 µg/ml	1.81 µg/ml
2	LOQ	3.0 µg/ml	3.06 µg/ml	6.0µg/ml

Table No: 9

System suitability parameters (HPLC)

S.No	Parameter	Paracetamol	Chlorzoxazone	Aceclofenac
1	Capacity factor	1.5	2.1	3.09
2	Asymmetry factor	1.09	1.12	1.16
3	Theoretical plates/meter	24648	69662	76659
4	Linearity	100-500 µg/ml	100-500 µg/ml	20-100 µg/ml

TABLE NO: 10.

Linearity and range of aceclofenac (HPLC with internal standard)

S.No.	Concentration of aceclofenac in µg/ml	Peak area ratio
1	20	0.0668
2	40	0.1275
3	60	0.2031
4	80	0.2604
5	100	0.3305

TABLE NO : 11

Linearity and range of chlorzoxazone (HPLC with internal standard)

S.No.	Concentration chlorzoxazone in µg/ml	Peak area ratio
1	100	0.3621
2	200	0.7325
3	300	1.1060
4	400	1.4659
5	500	1.8210

TABLE NO: 12

Linearity and range of paracetamol (HPLC with internal standard)

S.No.	Concentration paracetamol in µg/ml	Peak area ratio
1	100	0.4106
2	200	0.8302
3	300	1.2596
4	400	1.6452
5	500	2.0414

TABLE NO: 13

Estimation of aceclofenac, paracetamol and chlorzoxazone
(HPLC with internal standard)

S.No	Name of the drug	Label claim (mg/tablet)	Amount found (mg/tablet)	standard deviation	Relative standard deviation
1	Aceclofenac	100	99.8985	0.6076	0.6042
2		100	100.7355		
3		100	101.0801		
1	Paracetamol	500	501.3742	0.8708	0.1740
2		500	500.104		
3		500	499.7071		
1	chlorzoxazone	500	496.8205	0.7363	0.1480
2		500	497.0013		
3		500	498.1767		

TABLE NO:14

Recovery of aceclofenac, paracetamol and chlorzoxazone

(HPLC with internal standard)

S.No	Drug	Amount of drug in sample (mg)	Amount of drug added (mg)	Amount Recovered (mg)	Percent Recovery
1	Aceclofenac	99.8985	10	9.8963	99.96304
		100.7355	10	9.9947	99.9477
		101.0801	10	9.9455	99.4554
2	Paracetamol	501.3742	50	50.4113	100.8226
		500.104	50	50.2922	100.5845
		499.7071	50	49.9349	99.8699
3	Chlorzoxazone	496.8205	50	49.814	99.628
		497.0013	50	50.0143	100.0287
		498.1767	50	50.2463	100.4927

Table No: 16

Statistical parameters in HPLC (with internal standard)

S.No	Parameter	Aceclofenac	Paracetamol	Chlorzoxazone
1	Regression equation (y)	0.0033 x - 0.0003	0.0040 x + 0.145	0.0036 x + 0.0022
2	Slope (m)	0.0033	0.0040	0.0036
3	Intercept (b)	-0.0003	0.0145	0.0022
4	Correlation coefficient(r)	0.9993	0.9997	0.9999

Table No: 15

Precision data for aceclofenac, paracetamol and chlorzoxazone

(HPLC with internal standard)

S.No	Drug	Conc. Of the drug in µg/ml	Peak area	Standard deviation	C.V.
1 2 3	Aceclofenac	60	0.2028 0.2049 0.2044 0.2058 0.2035 0.2039 0.2068 0.2047	0.0012	0.6226
1 2 3	Paracetamol	300	1.2614 1.2601 1.2567 1.2601 1.2604 1.2599 1.2610 1.2608	0.0015	0.1212
1 2 3	Chlorzoxazone	300	1.1057 1.1051 1.1057 1.1056 1.1073 1.1089 1.1073 1.1064	0.0012	0.1138

Table No: 17

LOD and LOQ (HPLC with internal standard)

S.No	Parameter	Aceclofenac	Paracetamol	Chlorzoxazone
1	LOD	0.91 µg/ml	0.89 µg/ml	1.82 µg/ml
2	LOQ	3.1 µg/ml	3.03 µg/ml	5.9µg/ml

Table No: 18

System suitability parameters (HPLC with internal standard)

S.No	Parameter	Paracetamol	Atorvastatin	Chlorzoxazone	Aceclofenac
1	Capacity factor	1.5	1.8	2.1	3.09
2	Asymmetry factor	1.09	1.02	1.12	1.16
3	Theoretical plates/meter	24548	46839	69693	76793
4	Linearity	100-500 µg/ml	-	100-500 µg/ml	20-100 µg/ml

RESULTS AND DISCUSSION

The separation of aceclofenac, paracetamol and chlorzoxazone by HPLC method has been carried out in two ways. The first method was performed without being using the internal standard while the second method has been performed by using the internal standard. Since the drugs were polar in nature, reverse phase chromatographic method has been developed. In both the methods the separation has been carried out by using the phenomenex ODS C18 column.

Various solvent systems has been tried in order to obtain adequate resolution and good peak shape. The development of the chromatogram using the mobile phase methanol and water (75:25) at pH 4.5 results in good resolution between the peaks but the peak of aceclofenac is more asymmetric.

The separation using the same mobile phase at pH 3 results in good resolution but the peak shape of aceclofenac is still asymmetric. The mobile phase was then changed to acetonitrile and water (60 : 40 pH 3) results in good peak shapes for all the three drugs as evidenced by the asymmetric factor, but the resolution between paracetamol and aceclofenac is not good.

The polarity of this mobile phase was then slightly decreased by increasing the concentration of acetonitrile (acetonitrile: water 65:35 pH3) which results in good resolution between three peaks and the peak shape is more symmetric as evidenced by the resolution and asymmetric factor.

The calibration curve was linear in the range by 20-100 µg per milliliter for aceclofenac and 100-500 µg per milliliter for paracetamol and chlorzoxazone. Further the co-relation co-efficient for aceclofenac is 0.993, paracetamol is 0.9999 and chlorzoxazone is 0.9999.Proved good linearity between concentration and area.

A robust and sensitive method was developed for the analysis of drugs in formulation by HPLC.

Atorvastatin was chosen as the internal standard for the estimation of aceclofenac, paracetamol and chlorzoxazone. Atorvastatin was selected based on its polarity and its absorption in the UV region. The solvent system acetonitrile: water 65:35 at pH3 provides adequate resolution and good peak symmetry as evidenced by the resolution and symmetric factor.

The calibration curve was linear in the range by 20-100 µg per milliliter for aceclofenac and 100-500 µg per milliliter for paracetamol and chlorzoxazone. Further the co-relation co-efficient for aceclofenac is 0.9993, paracetamol is 0.9997 and chlorzoxazone is 0.9999.Proved good linearity between concentration and area.

The comparison of the statistical parameters between both of this estimation showed that there is no significant difference between these methods. The results indicate that the developed method is highly robust and can be used without the internal standard.

The advantages of the developed HPLC method includes the isocratic separation of all the three drugs and the use of methanol for the extraction which has the UV cut off less than 210 nm results in good recovery. Also the developed method is fast and specific with no interference with the adjectives in the formulation.

HPTLC.

In HPTLC method, different mobile phases were tried in order to attain adequate resolution. The separation using chloroform, methanol and ammonia (48:11:05) results in poor resolution between aceclofenac and paracetamol as evidenced by the R_f values of 0.60 and 0.62.

The chromatogram was then developed using the mobile phase toluene, ethyl acetate and glacial acetic acid in the ratio of 15:10:05 results in a poor resolution between paracetamol (R_f =0.17) and chlorzoxazone(R_f =0.3).

The polarity of the mobile phase is then decreased by increasing the concentration of toluene (toluene, ethyl acetate and glacial acetic acid in the ratio of 15:10:0.2) was tried which produces good resolution aceclofenac (0.30) and chlorzoxazone (0.72) but the resolution between the paracetamol (0.19) and aceclofenac is (0.30).

The polarity and pH of the mobile phase is then decreased and the chromatogram is developed by using the mobile phase toluene, ethylacetate and glacial acetic acid in the ratio of (17.5:10:05) results in good separation between the drugs paracetamol, aceclofenac and chlorzoxazone as evidenced by the R_f values 0.12, 0.29, 0.72 respectively. This mobile phase was then taken for the further studies.

The calibration curve was linear in the range of 0.1mg/ml to0.5mg/ml for aceclofenac and 0.5mg/ml to 2-5mg/ml for paracetamol and chlorzoxazone. The correlation coefficient 0.9979, 0.9999 and 0.9998 for the drugs indicates good linearity between the concentration and area. The amount of aceclofenac, paracetamol and chlorzoxazone

estimated by this method is in good with label claim. The method allows reliable quantification of aceclofenac, paracetamol, and chlorzoxazone up to respectively. Statistical analysis proves that the method is repeatable, selective and accurate for the estimation of the drugs in the formulation.

UV-DIFFERENCE SPECTROSCOPY:

In difference spectrometry the UV-spectrum of aceclofenac is measured in 0.1M hydrochloric acid and 0.1M sodium hydroxide. The λmax of aceclofenac occurs at 272nm in 0.1M hydrochloric acid whereas the peak is shifted to 218nm in case of 0.1M sodium hydroxide. The difference absorbance is measured at 272nm since better linearity is obtained at this wavelength.

The difference spectrometric absorbance was linear in the range of 0.96µg/ml to96µg/ml. further the co-relation co-efficient of for aceclofenac shows good linearity between the concentration and differential absorbance (ΔA).

The results from the analysis of market sample reveals that the proposed method is in good agreement with the labeled amount. The recovery percentage from to were satisfactory which showed the reliability and suitability of the proposed method.

SUMMARY AND CONCLUSION

The formulation containing aceclofenac 100mg, paracetamol 500 mg and chlorzoxazone 500 mg were estimated by RP-HPLC and HPTLC methods. The chromatographic conditions like detection wavelength, the composition of mobile phase, the pH of the mobile phase were optimized for the best possible separation of these drugs in the formulation. The drug formulation is then estimated using the optimized chromatographic conditions and the comparative datas were given in the table.

Aceclofenac was estimated by differential spectropphotometric method. The results obtained by these methods including recovery studies were comparable which proves the repeatability and suitability of the methods.

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